Sónia Rodrigues Nóbrega

Licenciada em Bioquímica

Formulation of Chitin-glucan complex and FucoPol biopolymers particles for controlled release of fertilizers

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientadora: Doutora Cristiana Andreia Vieira Torres, Post-doc Researcher, FCT-UNL

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2017

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Palavras-chave

Complexo quitina-glucano (CGC), FucoPol, partículas, libertação controlada de fertilizantes, perfil de libertação, crescimento de sementes.

Resumo

O uso de fertilizantes de libertação controlada (FLC) na agricultura tem ganho cada vez mais interesse, pois permite torna-la num sector mais sustentável, diminuindo as perdas de nutrientes e aumentando a produtividade. A maioria dos FLC usa polímeros sintéticos como revestimento de modo a melhorar as suas propriedades. No entanto, estes polímeros como não são biodegradáveis, o seu uso acaba por causar um problema ambiental. Assim sendo, o uso de biopolímeros aparecem como uma boa alternativa para ultrapassar os problemas ambientais, uma vez que são biodegradáveis e não tóxicos. Este trabalho teve como objetivo produzir partículas de complexo quitina-glucano (CGC) e FucoPol, enriquecidas em azoto. Para tal, nitrato de amónia foi adsorvido em 15 g L^{-1} de partículas de CGC, numa solução saturada, em 2 h de contacto a pH 5.0. A adsorção de azoto foi de 22.55 %. Relativamente ao FucoPol, as partículas de hidrogel foram produzidas a partir de uma solução de polímero 10 g L^{-1} dissolvido em duas soluções de 15 e 30 g L^{-1} de nitrato de amónia, a qual foi deixada cair gota a gota numa solução de cloreto de ferro (III) heptahidratado (3.30 g L^{-1}). As partículas encapsularam 0.34 g L^{-1} e 0.53 g L^{-1} de azoto, respetivamente.

O perfil de libertação de azoto, de ambas as partículas, em água e no solo, foi estudado. O perfil de libertação das partículas de CGC em água a 25 ºC, verificou-se uma libertação prematura da amónia (NH_4^+) e nitrato (NO_3^-) nos primeiros dois dias de ensaio, e no solo também nos primeiros cinco dias de ensaio. As partículas de FucoPol na água foi observado uma libertação prematura de amónia e nitrato no primeiro dia, e no solo em dois dias. A água é um meio mais extremo que o solo, que potenciaria a libertação abrupta dos nutrientes das partículas.

Por fim, as partículas de biopolímeros enriquecidas em azoto foram avaliadas na germinação e crescimento de sementes de ervilha (*Pisum sativum*). O ensaio com partículas de FucoPol como adubo apresentou uma boa germinação e um crescimento elevado da planta, demonstrando que as partículas de FucoPol têm potencial para serem usadas na libertação controlada de nutrientes. Relativamente às partículas de CGC estas apresentaram um crescimento mais lento.

Keywords

Chitin-glucan complex (CGC), FucoPol, particles, controlled release fertilizer, release profile, seeds growth.

Abstract

The development of controlled release fertilizers (CRFs) as increased interest for the agriculture sector, in order to turn it in a more sustainable activity, diminishing nutrient losses and increasing crop productivities. The CRFs properties are improved with the use of coatings, which is mostly performed using synthetic polymers, but since they are not biodegradable causing an additional environmental concern. In order to overcome this issue synthetic problems may be substituted by biopolymers, which are biodegradable and non-toxic like chitin-glucan complex and FucoPol.

The main goal of this work was to produce nitrogen containing particles from two microbial biopolymers, chitin-glucan complex (CGC) and FucoPol. Therefore, ammonium nitrate was adsorbed to CGC particles at 15 g L^{-1} by submitting them to a saturated solution. There was a nitrogen uptake of 22.55 %, in 2 h contact, at pH 5.0. Hydrogel particles were produced from a FucoPol 10 g L^{-1} solution dissolved in 15 and 30 g L^{-1} of ammonium nitrate, and dropped in iron (III) chloride hexahydrate solution. The nitrogen uptake was 0.34 g L^{-1} and 0.53 g L^{-1}, respectively.

The nitrogen particles release was tested in water and soil. For the nitrogen CGC particles in water at 25 ºC, a burst release was verified for ammonium (NH_4^+) and nitrate (NO_3^-) in the initial two days assay and in soil, the same happened in the initial five days assay. To the nitrogen FucoPol particles in water was observed a burst release for ammonium and nitrate in the first day and in soil in two days. The water is an extremer medium than soil, which would potentiate the burst release of the nutrients from the particles.

Lastly, the nitrogen biopolymer particles were evaluated in the germination and growth of pea (*Pisum sativum*) seeds. For the tests with nitrogen FucoPol particles as fertilizer it was verified a good germination and fast growth. Tests with nitrogen CGC particles presented a slower germination and growth. Therefore, nitrogen FucoPol particles have potential to be used in controlled release of nutrients.

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Abbreviations

AIM Alkaline insoluble material

CGC Chitin-glucan complex

CRFs Controlled release fertilizers

EEFs Enhanced efficiency fertilizers

EPS Exopolysaccharide

HPLC High performance liquid chromatography

SEM Scanning electron microscopy

SRFs Slow release fertilizers

TFA Trifluroacetic acid

1. Introduction

1.1. Crop Production and Fertilizers

The world population has increased very rapidly in the last years, causing the food production to increase also, and this demands an increase in crop production (Davidson and Gu, 2012; Kashyap *et al.*, 2015).

An intense crop production leads to a depletion of soil nutrients. In order to reuse the soil and ensure the quantity and quality of the crop produced, it is enriched with nutrients by the application of fertilizers. Fertilizers are chemical substances that are added to the soil for healthy growth of plants and, consequently, increase the crop yield. The main components of fertilizers are nitrogen (N), potassium (K) and phosphorus (P) (Davidson and Gu, 2012; Timilsena *et al.*, 2014).

However, only a small amount of the fertilizer applied in the soil is really used by the plants, so the main amount is lost to the environment, this leads to a waste of nutrients. Nitrogen is lost by leaching, mineralization, erosion and denitrification processes. Phosphorus is lost by surface run-off and mineralization. Potassium lost is due to leaching and surface run-off (Davidson and Gu, 2012; Timilsena *et al.*, 2014).

Nutrient loss in large amounts and consequential transformation processes also leads to environmental pollution problems, like contamination of ground water, soil acidification, eutrophication, accumulation of heavy metals and formation of persistent organic pollutants (Davidson and Gu, 2012; Timilsena *et al.*, 2014).

To practice a sustainable agriculture, a new type of fertilizers has been developed, the enhanced efficiency fertilizers (EEFs), that promote a controlled release of the nutrients that are needed for the healthy plant growth, in smaller quantity that reduces nutrient loss, and reduce the environmental pollution (Davidson and Gu, 2012; Timilsena *et al.*, 2014).

The enhanced efficiency fertilizers can be divided into three groups; the chemically modified slow release fertilizers, the controlled-release fertilizers with barrier layer and the enhanced efficiency fertilizers containing inhibitors (Timilsena *et al.,* 2014).

Slow release fertilizers (SRFs) are fertilizers in which nutrient release rate is slower than in a traditional fertilizer, but parameters like the rate, duration, and pattern of nutrient release aren`t controllable and completely predictable. In controlled release fertilizers (CRFs) these parameters are defined during the production and, so, predictable and controllable (Trenkel, 2010).

In the SRFs, the nutrients are uncoated and the release depends on the soil properties, for example, temperature, pH, moisture, and microorganism's activity. This class of fertilizers doesn`t decrease the germination and doesn`t destroy the vegetation. However, the crop yield doesn`t improve, and the nutrients release is unpredictable. In this group there is UF (urea-

formaldehyde), IBDU (isobutyliden diurea), UT (urea-triazone), $MgNH_4PO_4$ (magnesium ammonium phosphates) and PAPR (partially acidulated phosphate rocks) (Shaviv, 2000; Timilsena *et al.,* 2014).

For example, the urea-formaldehyde (UF) is composed by two fractions, a low molecular weight and a high molecular weight. This slow release product had a problem, the low molecular weight fraction releases too much nutrients than the required for an early stage of plant grow, and the high molecular fraction releases too low nutrients. The UF release depends much on microbial action and on other soil properties such as pH, temperature and humidity (Shaviv, 2000; Timilsena *et al.,* 2014).

In the controlled-release fertilizers with a barrier layer, the nutrients release can be more effectively controlled, delaying chemical and microbial action, and depends on permeability to water, solubility of the core and matrix degradability. As barrier layer, there are polymers, natural polymers like alginate, lignin and chitosan, or synthetic polymers like polyacrylamide, polystyrene, and polyvinyl chloride. The synthetic polymers are better on controlling the release properties, but they are more difficult to degrade on the soil originating residual accumulation, which is a pollutant. Natural polymers are biodegradable and non-toxic, however they have poor control over nutrient release and their production is more expensive, disadvantages that have been worked on to overcome (Timilsena *et al.,* 2014). Still, natural polymers are gaining acceptance over the synthetic polymers for controlled-release formulations because of their eco-friendly nature, cost effectiveness, easy availability, and biodegradability (Campos *et al.*, 2015).

Lastly, the enhanced efficiency fertilizers containing inhibitors consists in the application of substances that inhibits the microbial and chemical action over the nutrients, preventing the loss and transformation of the nutrients. It is considered the most efficient of the EEFs, and the most economically viable. In this group, the most used are nitrification inhibitors and urease inhibitors. For example, the nitrification inhibitors avoid the nitrification process by interfering in the nitrifying bacteria metabolism, which retains the nitrogen in the ammonium form which is less volatilized (Timilsena *et al.,* 2014).

Another type of coated fertilizers has been developed, they have super-absorbent properties, where the idea is to slow the release of the nutrients because of the water content that is going to decrease the diffusion. As coating material is used hydrophilic polymers. The disadvantage in this case is that they absorb water during storage that is going to dilute the nutrient concentration, and so to fill this gap, is necessary to use more quantities of nutrients (Timilsena *et al.,* 2014).

An uncoated enhanced efficiency fertilizer has been developed recently and has showed better results than the coated fertilizers. The fertilizer super-granules are applied in the soil and the release of nutrients lasts for a long time (Timilsena *et al.,* 2014).

The ideal nutrient release pattern for a fertilizer should release nutrients according with the plant growing necessities, as it can be seen in Figure 1.1. SRFs and CRFs could accomplish this ideal fertilizer profile (Trenkel, 2010).

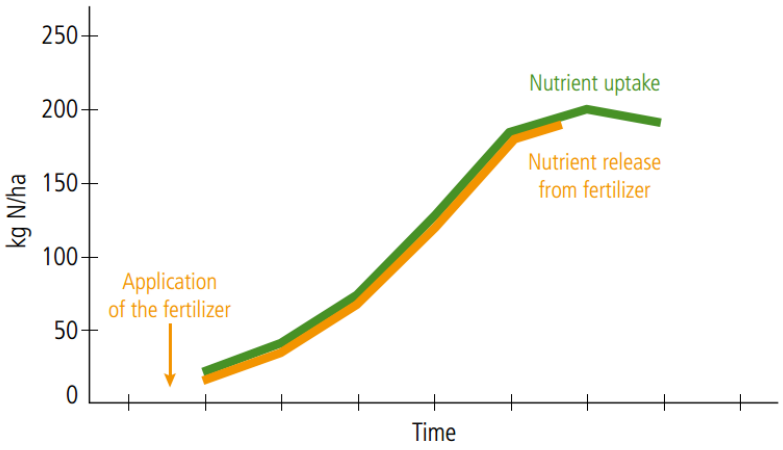

Figure 1.1. Ideal nutrient release pattern from a fertilizer (adapted from Trenkel, 2010).

1.2. Future Perspectives on Fertilizers Use in the World

An outlook for world fertilizer trends was performed by the Food and Agriculture Organization of the United Nations (FAO) in 2015. It was estimated that until 2018 the total fertilizer nutrient ($N + P_2O_5 + K_2O$) demand in the world would be 200 500 000 tons, an estimative that the fertilizer demand would grow 1.8 % per year in the period between 2013 and 2018 (Figure 1.2). This estimative was performed based on the global economic growth situation, where after a period of financial instability since 2008, tended to stabilize and grow during 2014-2015. Another point in favor of fertilizer demand is that with the increasing energy costs and the increasing production and availability of shale gas in the USA will decrease the natural gas costs, and so in a near future it is expected that fertilizer global industry will be based on natural gas (FAO, 2015).

World nitrogen fertilizer is expected to have a 1.4 % annual growth (119 400 000 tons in 2018) since the growth rate between 2013 and 2014 was of 1.5 %. Between 2014 and 2018 of the total nitrogen demand 58 % would be in Asia, 22 % in the American continent, 11 % in Europe, 8 % in Africa and 1 % in Oceania (FAO, 2015).

For phosphate demand it is expected to have a 2.2 % growth rate per year (46600000 tons in 2018) since the growth rate between 2013 and 2014 was of 2.4 %. Between 2014 and 2018 of the total phosphate demand 58 % would be in Asia, 29 % in America, 9 % in Europe, 4 % in Africa and 0.5 % in Oceania (FAO, 2015).

Finally, the world potassium fertilizer demand is expected to have a 2.6 % growth rate per year (34 500 000 tons in 2018), since the growth rate between 2013 and 2014 was of 3.3 %. Between 2014 and 2018 of the total potassium demand 56 % would be in Asia, 27 % in America, 11 % in Europe, 6 % in Africa and 0.4 % in Oceania (FAO, 2015).

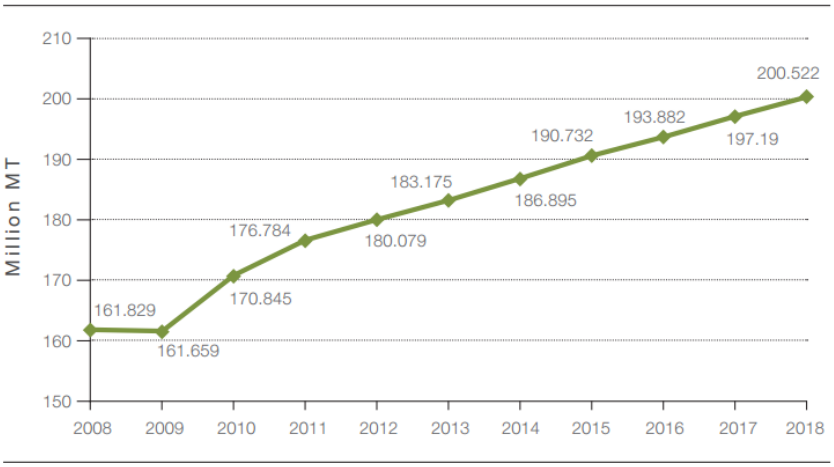

Figure 1.2. Global nutrients ($N+P_2O_5+K_2O$) consumption tendency until 2018 (adapted from Trenkel, 2010)

These estimates are depended on several variables that could change the outlook, like weather changes or plagues that could put in jeopardy the crop production success, changes in agriculture merchandise costs, biofuel prices evolution, legislation evolution and world economic evolution (Heffer and Prud`homme, 2015).

1.3. Legislation

There is no universal legislation to regulate the slow and controlled release fertilizers market, which would serve to prevent the consumer from being misled to buy fertilizers that don`t have the promised slow or controlled release properties.

According to Trenkel, in 1995, Israel proposed the registration of slow and control release fertilizers to include information on the expected nutrient release pattern, possible factors that could affect the release, and the release mechanisms (Trenkel, 2010).

In the European Union, regulation on fertilizers products was proposed to be reviewed to adapt to new issues on this field, including the controlled release fertilizer regarding to the coating biodegradability. The EU Comission proposed that the polymer coating of the controlled release fertilizer should in 24 months maximum (at 25 ± 2 ºC) be degraded in 90 %. The organization Fertilizers Europe didn`t find this proposal realistic for the existing polymer coating in the market, they found to be a fast degradation requirement and would go against the working CRFs fundamental (Fertilizers Europe, 2016).

Testing on release profile on slow and control release fertilizers are performed on water (at 25 ºC) and on soil and determine the time that is required to 80 % of nutrients to be released (Trenkel, 2010).

The European Standardization Committee (CEN) Task Force on Slow-Release Fertilizers proposed that to be considered a slow release fertilizer it should, at 25 ºC, release no more than

15 % of the total nutrient content in 24 h and no more than 75 % in 28 days (Trenkel, 2010; Roshanravan *et al.*, 2015). Further, the release rate of the nutrients should be slower than the release rate from a traditional fertilizer, where the nutrients are directly available (Trenkel, 2010).

1.4. Polysaccharides

Polysaccharides are macromolecules composed of long chains of monosaccharides units bonded by glycosidic linkages, and they are diversified in their chemical properties, since they are varying in their chemical composition (Liu *et al.*, 2008; Raemdonck *et al.*, 2013; Campos *et al.*, 2015).

These macromolecules are present very abundantly in nature, and so they have a good availability, they are biocompatible, biodegradable, and non-toxic, characteristics that makes these polymers valuable for biomedical and pharmaceutical applications (Liu *et al.*, 2008; Raemdonck *et al.*, 2013; Campos *et al.*, 2015).

Due to the various functional groups (amines, carboxylic acid, hydroxyls for example) in the glycosidic units, polysaccharides can be easily chemically modified to form derivatives with specified functions. In addition, their processing is low-cost, being advantageous for large scale production of their derivatives (Liu *et al.*, 2008; Raemdonck *et al.*, 2013; Campos *et al.*, 2015).

Some of the polysaccharides such as alginate, cellulose, pectin, cyclodextrin, starch, dextran, chitosan and guar-gum have been used for control-release applications for agrochemicals (pesticides, herbicides and fertilizers). These polymers were used in different forms, such as spheres, microspheres, nanoparticles and hydrogels to carry and perform the controlled-release of the agrochemicals (Campos *et al.*, 2015).

1.5. Chitin-glucan Complex (CGC)

Chitin-glucan complex (CGC) is a co-polymer cell wall component, maintains its integrity, that can be found in most fungi and yeast, for example in *Arpergillus niger*, *Saccharomyces cerevisiae* and *Komagataella pastoris* (previously known as *Pichia pastoris*) (Farinha *et al*., 2015).

The CGC molecular structure consists on a covalent linkage between chitin (N-acetyglucosamine units) and 1,3-glucans polymers (units of glucose) (Figure 1.3). This polymer is water insoluble and also insoluble in most organic solvents, is hygroscopic and presents a high swelling capacity. In addition, it has biocompatible, biodegradable, anti-oxidant, antibacterial and non-toxic properties, making an interesting and valuable biopolymer for applications for biomedicine or food industry. Another advantage of this co-polymer, is that its production and extraction doesn`t include an animal source, and can be a chitin alternative source, which traditional extraction from shelves included strong acids or bases for demineralization and deproteination, that are toxic for the environment (Roca *et al.*, 2012; Farinha *et al.*, 2015, 2016).

CGC was successfully produced and extracted from *Komagataella pastoris* using as only carbon source glycerol byproduct from the biodiesel industry in the culture medium, an inexpensive raw material. This methylotrophic yeast as two principal advantages, during fermentation has the capacity to reach high cellular densities and to reach high product yields (Roca *et al.*, 2012; Farinha *et al.*, 2016).

A purified CGC had a β-glucan:chitin molar ratio content of 75:25, presented a low protein (3 wt%) and inorganic salts (0.9 wt%) presence, and had an high average molecular weight ($4.9x10^5$ Da) (Farinha *et al.*, 2015). It was reported that polymer β-glucan:chitin molar ratio could be manipulated with pH and temperature cultivation variations, where the highest molar ratio obtained (>14:86) was in the pH range 4.5-5.8 and in the temperature range of 26-33 ºC, so this biopolymer production can be manipulated to achieve a higher chitin content (Chagas *et al.*, 2014).

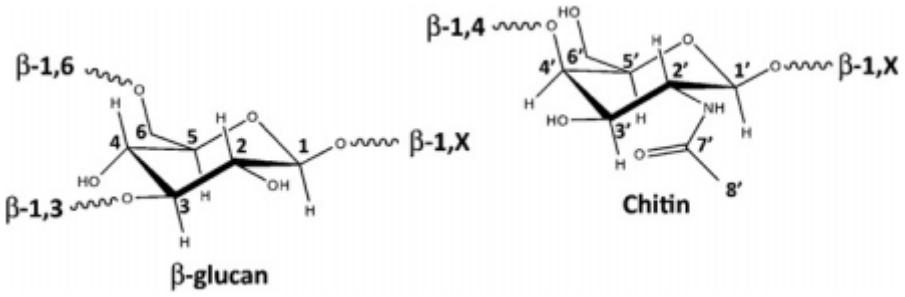

Figure 1.3. β-glucan and chitin chemical structure representation (adapted from Roca *et al.*, 2012).

1.6. FucoPol

It was found that the bacterium *Enterobacter* A47 (DSM 23139) secretes a fucose-containing exopolysaccharide (EPS), that was named FucoPol (Alves *et al.*, 2010; Freitas *et al.*, 2014).

Exopolysaccharides (EPS) are microbial polysaccharides that are found in the cell surface. EPS are composed of sugar monomers, being the most common glucose and galactose, and non-sugars components as acyl groups (Torres *et al.*, 2011).

The bacterium *Enterobacter* A47 has been reported to produce polymers with different composition when subjected to different pH and temperature conditions and to different carbon sources in cultivation medium (Freitas *et al.*, 2014).

FucoPol is composed of the sugars fucose (32-36 mol%), galactose (25-26 mol%), glucose (28-37 mol%), glucuronic acid (9-10 mol%), by the acyl groups succinyl (2-3 wt.%), pyruvyl (13-14 wt.%), and acetyl (3-5 wt.%). This polymer has a high molecular weight ($5x10^6$ Da), and after purification, the protein content is below 5 wt.% (Alves *et al.*, 2010; Torres *et al.*, 2011, 2015).

The characterization of FucoPol demonstrated that it has flocculating capacity, emulsifying and rheological properties that could be interesting for a variety of applications including cosmetic and pharmaceutical industry (Freitas *et al.*, 2011, 2014).

1.7. Polymer Hydrogel

Polymer hydrogels are defined as polymeric networks that have a great absorbing capacity of water in the structure, and in aqueous solution doesn`t dissolve. Normally these hydrogels formation can be performed at the ambient temperature and without resource to organic solvents. These hydrogels have been formulated for biomedical applications, including drug-delivery systems (Lin and Metters, 2006).

Hydrogels have been used for soil irrigation, to increase the water retention in arid soils to promote plant growth without wasting water, for example (Montesano *et al.*, 2015). These hydrogels have been also formulated for controlled-release fertilizers, for example a chitosan hydrogel for potassium release and water retention (Jamnongkan and Kaewpirom, 2010), and chitosan hydrogel for slow-release of dicyandiamide, a nitrification inhibitor (Minet *et al.*, 2013). In polyacrylamide gels, it was mentioned that the fertilizers salts reduced the hydration and altered the gel physical properties (Montesano *et al.*, 2015).

Other type of gel used for water retention for soil irrigation are the super absorbent hydrogels polymers, they are hydrophilic polymers that have the capacity to absorb and retain water up to thousands of times their weight in a short period of time, and about 95 % of the water content can be available for plant absorption (Vundavalli *et al.*, 2015).

It was referred that the majority of hydrogels in the market are acryl-based products that aren`t biodegradable and so a pollutant for soil (Montesano *et al.*, 2015). Polysaccharides are an attractive alternative to be used as hydrogels mainly because of their biodegradability, availability and non-toxicity. But this biodegradability could cause a faster release of nutrients than requested by the plant growth requirements. An alternative to maintain through time the gel matrix stable and maintain the gel properties is through chemical crosslinking, for example, by the incorporation of vinyl groups that has been widely used (Guilherme *et al.*, 2015).

1.8. Adsorption

Adsorption is a surface phenomenon that involves the interaction of one or more solutes from an aqueous phase and a rigid phase (Wong *et al.*, 2004).

In this work, uptake of the ammonium salts by the CGC polymer will be performed by adsorption, since this polymer as a high swelling capacity as referred earlier (Farinha *et al.*, 2015). Since this procedure has never been done with this polymer, research on adsorption properties

of chitin and chitosan is the much closer to try to extrapolate and adapted the methods and conditions for CGC optimum adsorption capacity.

In a study on effluents treatment from the textile industry (Du *et al.*, 2008), chitosan nanoparticles were used to remove a model anionic dye, eosin Y, from an aqueous solution. Variable parameters where studied to see changes on adsorption capacity, they were the concentration of dye and polymer, pH, temperature, and time of contact between dye solution and polymer (Du *et al.*, 2008).

In the pH effect, the chitosan adsorption capacity between pH 2.0 and pH 5.0 tended to decrease but slightly, and when reach pH 7.0 the adsorption capacity had an abrupt decrease. It was explained that the interaction between the chitosan protonated amino groups and the eosin Y anionic group is what causes the adsorption between them. With the increase of the pH value the protonated amino groups decrease, causing the interaction between polymer and dye decrease, and consequently the adsorption capacity. Desorption phenomenon could be observed between pH 10.0 to pH 12.0 (Du *et al.*, 2008).

Chitosan beads were studied to uptake chromium from an aqueous phase (Ngah *et al.*, 2006), which the optimal pH was in the range pH 3.0-5.0, and the adsorption equilibrium time was at 50 min of contact (Ngah *et al.*, 2006).

In another study (Saha *et al.*, 2010), chitosan was used to adsorb methyl orange from aqueous solution, where the dye concentration (5-50 µmol L^{-1}), pH (4.0-9.0) and temperature (27-45 ºC) were tested. It was concluded that although with the temperature increase the adsorption capacity increased slightly, but was considered insignificant; the adsorption capacity increased with the pH decrease, and adsorption equilibrium was reached at 60 min for pH 4.0-5.0; the dye concentration increase lead to an increase in the adsorption capacity (Saha *et al.*, 2010).

Chitin as also been studied in dye removal. In a study (Akkaya et al., 2007) by using chitin as adsorbent for reactive yellow 2 (RY2) and reactive black 5 (RB5) dyes, the goal was to study the absorbance capacity of chitin variating factors conditions, such as pH, temperature, concentration and shaking rate. Using 0.2 g of chitin in 300 ppm and 600 ppm dye solutions, it was concluded that concentration and shaking rate effect wasn`t significant. For dye RY2, chitosan maximum adsorption capacity was obtained at lower pH and temperature, and for dye RB5 was obtained at higher pH and temperature (Akkaya *et al.*, 2007).

Other study used cellulose/chitin beads for adsorption of heavy metals (Zhou *et al.*, 2004) and verified that temperature variation (10-40 ºC) didn`t had a significant influence on adsorption capacity, pH 4.0-5.0 was the range that demonstrated the higher adsorption capacity, and the time of contact to reach adsorption equilibrium was 4-5 h (Zhou *et al.*, 2004).

1.9. Nutrient Release Methodology

According to Kashyap *et al.* (2015), in agricultural release systems there are two release systems that can occur simultaneously for chitosan particles, by diffusion or degradation. The diffusion release stars with the water penetration in the polymer matrix making it swell, then the internal structural polymer changes and gets malleable and then the nutrients diffuses out of the matrix. The degradation release can occur by many factors, as erosion, chemical reactions or by microbial action. A burst release by osmosis can also occur when the nutrients are adsorbed to the particles surface (Kashyap *et al.*, 2015).

To test the nutrient release profile of the CRFs, they are tested in water an in soil. Mayer (2010) tested commercial CFRs in water, using 10 g of CRFs in 250 mL of water that was collected and exchanged every day in a temperature controlled environment, at 25 ºC and at 100 ºC to accelerate the release process, during 180 days. In soil was used 3 g of CRFs in vases with and without plants, irrigated with 250 mL of water every day during 180 days at a greenhouse (Mayer, 2010).

Trinh *et al.* (2014; 2015) used CRF commercial Agrium ®coated urea for nitrogen release, testing in water used 2 g of the CRF in a beaker with 250 mL of water at 27 ºC, where the urea concentration was measured by the spectrophotometer at 210 nm in a period of time of 2-5 days during 82 days, when the nitrogen reached the 99 %. At each collecting the total volume of water was replaced (Trinh *et al.*, 2014; 2015).

Hussain *et al.* (2012) used chitosan microspheres prepared by emulsification and cross-linked with genipin with urea encapsulated. The release tests were performed in 100 mL of water, and the refractive index was measured every day for 8 days. They tested different temperatures, 25 ºC, 30 ºC and 35 ºC, the release profile was similar between, but with temperature increase, the urea rate release increased also (Hussain *et al.*, 2012). Melaj and Daraio (2013) used layered matrix tablets of chitosan and xanthan with potassium nitrate encapsulated and studied its release in water in a static test and in a dynamic test with magnetic agitation (250 rpm). The test was performed in 500 mL water with the immersion of a tablet at 25 ± 2 ºC during 160 days and the potassium nitrate release was monitored by the conductivity. The dynamic test was used to accelerate the nutrients release, and the static test was closer to the soil experience (Melaj and Daraio, 2013).

Roshanravan *et al.* (2015) tested the release properties of urea-kaolinite bonded with chitosan in water, using 1 g in 200 mL of water at 25 ºC, and at each 5 days during 30 days 0.1 mL was collected and analyzed for the urea content using the diacetylmonoxim colorimetric method using UV-vis spectrometry (Roshanravan *et al.*, 2015). Sempeho *et al.* (2015) also tested urea-kaolinite but in the form of nanocomposites in water for six days, where a certain amount of nanocomposite was added to water contained in dialyses membranes with constant shaking. Urea content was measured every 5 h period by spectrometry using a hypochlorite and phenol method (Sempeho *et al.*, 2015).

Yamamoto *et al*. (2016) used a urea-formaldehyde polymer nanocomposite for urea release tests in water at 25 ºC sealed with parafilm to reduce the evaporation, and with gentle agitation using an orbital shaker. 1 mL sample were collected to analyze the urea content using UV-vis spectrometry during 4 days. They tested in soil too, varying the humidity (60 %, 70 % and 80 % at 30 ºC) and temperature (25 ºC, 30 ºC and 35 ºC with 60 % humidity) (Yamamoto *et al.*, 2016).

Li *et al.* (2016) tested the release properties of a wheat straw cellulose hydrogel with nitrogen and phosphorus encapsulated in water, using 1 g of sample in 1 L of water, with 2 mL sample collected regularly during 120 days for quantification of the nitrogen and phosphorus release by UV-vis spectrometry (Li *et al.*, 2016).

1.10. Motivation

To enhance crop productivity to keep up with the worldwide increasing demand for food, the agriculture sector has used fertilizers in excessive amounts, and this led to environmental pollution (Kashayp *et al.*, 2015). Controlled release formulations have been studied and produced for fertilizers applications, so it is only necessary one fertilizer application with the right quantity that will meet the crops nutritional demands (Timilsena *et al.*, 2014). These CRFs can be coated, in order to avoid a burst release when in contact with the soil elements, such as microbial activity. Some of the materials used for the coating are synthetic polymers that have good controlled release properties, but are of difficult biodegradability, creating an additional environmental problem with residues accumulation in soil. Biopolymers are an alternative, are biodegradable and non-toxic (Timilsena *et al.*, 2014; Campos *et al.*, 2015).

The goal of this thesis work was from chitin-glucan complex (CGC) and FucoPol, two biopolymers with microbial origin and low-cost production from raw-materials, to produce particles for controlled release of nitrogen, and test its release behavior in water, in soil and its effect in the germination and grow in pea seeds (*Pisum sativum*).

2. Material and Methods

2.1. CGC Extraction

Extraction of Chitin Glucan Complex (CGC) from the fermentation broth (2260 mL) of *Komagataella pastoris* was performed by an alkaline treatment with 1 M of NaOH (eka) at 65 ºC for 2 h for deproteinization of the cell wall components.

After cooling the suspension, it was diluted 2 times with deionized water and centrifuged (8000 rpm for 10 min) (Sigma 4-16KS). The alkaline insoluble material (AIM) was resuspended in deionized water, neutralized with HCl 1 M and centrifuged again as described above. Then, it was centrifuged again, and this process was repeated until the conductivity (Mettler Toledo) of the extract was below 200 µS cm^{-1}. Conductivity and pH (VWR) parameters were verified and controlled during the process.

Then, the CGC samples were dried in a stove (Cassel) at 70 ºC for 24 h. The dried CGC was weighed in a semi-analytical scale (VWR) and milled with a coffee grinder (Selecline) for further analysis.

2.2. CGC Properties Analysis

2.2.1. Ash Content

To quantify the CGC inorganic salts content, 0.5 g of milled CGC was weighted in a previously weighted porcelain crucible and placed in a muffle furnace at 550 ºC overnight. The ashes remaining in the crucible were weighted and represented the inorganic content of the CGC.

2.2.2. Sugar Analysis Composition

For CGC analysis composition, the samples were subjected to two hydrolyzes. The first with trifluoroacetic acid (TFA) (Sigma-Aldrich) to hydrolyse the β-glucan fraction of the polymer. A stronger acid (HCl) (Sigma-Aldrich) was imposed for the quantification of the chitin fraction. Dried CGC samples (5 mg) were resuspended in 5 mL deionized water followed by the addition of 100 µL of TFA. Then, samples were hydrolyzed for 2 h at 120 ºC in a heating block (Stuart SBH 130D).

For HCl hydrolysis, 5 mg of CGC were added to 5 mL of 4 N HCl and hydrolyzed for 5 h at 120 ºC in a digester (standard solution: 5 mg of glucosamine and 5 mL of 4 N HCl solution). After hydrolyses, samples were cooled and transferred to 5 mL sample tubes. A 500 µL sample was transferred back to the tube at 120 ºC to evaporate the solvent, this process took about 2 h. After the solvent was evaporated, the tubes were cooled and 1 mL of distilled H_2O was added to the tube (1:2 dilution) and resuspended in the vortex, filtered and transferred to HPLC vials (700 µL).

Both hydrolysates were used for the quantification of the constituent monosaccharides by HPLC, using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed at 30 °C, with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 mL min^{-1}. Glucose and glucosamine were used as standards in a range of concentrations between 1 and 0.005 g L^{-1}. Standards were subjected to the same hydrolysis procedure as the samples.

2.3. FucoPol

2.3.1. FucoPol Sugar Analysis

For FucoPol sugar composition analysis, 3 mg of dried samples (with replica) were dissolved in 5 mL of deionized water hydrolysed with 100 µL of TFA for 2 h at 120 ºC. Samples were filtered and transferred to HPLC vials for analysis (700 µL sample).

The acid hydrolysate was used for the identification and quantification of the constituent monosaccharides by High Performance Liquid Chromatography (HPLC), using a CarboPac PA10 column (Dionex), equipped with an amperometric detector (Dionex). The analysis was performed at 30 ºC, with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 mL min^{-1}. Galactose (Fluka), mannose (Fluka), rhamnose (Fluka), glucose (Scharlau), fucose (Sigma) and glucuronic acid (Alfa Aesar) solutions (0.1 – 0.005 g L^{-1}) were used as standards.

2.4. Nitrogen CGC Particles

Nitrogen CGC particles were produced by resuspending milled CGC in a saturated ammonium sulphate solution (Applichem), for 24 h with magnetic stirring. Several CGC concentrations (1.5; 5.0; 7.5; 10 and 15 g L^{-1}) were tested.

Higher swelling was observed for a CGC concentration of 15 g L^{-1}. Therefore, further absorption tests with CGC concentrations of 15, 20, 25 and 30 g L^{-1} were performed.

Besides ammonium sulphate, three other nitrogen salts were tested for absorption capacity of CGC particles, namely potassium nitrate (Sigma-Aldrich), ammonium nitrate (Fluka) and urea (Harnstoff). First, it was necessary to saturate the solutions, by adding small quantities of the salt into 100 mL of deionized water, with magnetic stirring. For ammonium sulphate it was necessary to use 72.32 g to reach the saturation point, 122.22 g for saturation of urea solution, 30.96 g for saturation of potassium nitrate solution and 180.60 g for saturation of ammonium nitrate solution. Then, the saturated solutions were filtered with a 0.45 µm nylon syringe filter (Wathman).

The absorption tests were performed during 48 h. Samples were taken at time zero (t0), 1, 5, 24, 30 and 48 h. At each sampling, 6 mL were collected and centrifuged at 8000 rpm at 20 ºC for 15 min. The supernatant was separated from the pellet and stored at -20 ºC. The pellet was also stored at -20 ºC and further dried in a stove at 60 ºC. At the end of the assays, the nitrogen CGC particles were collected and dried in a stove at 60 ºC.

Before adding the CGC, 2 mL of each salt solution were collected and stored for further analysis.

The urea and ammonium nitrate solutions precipitated after the ending of the first assay with the CGC particles. Further solutions of these two salts were made with half of the concentration in order to prevent future solution precipitation.

A blank assay was performed with deionized water for the various CGC concentrations, using the same conditions used for the salts.

2.4.1. Effect of pH in the Absorption Capacity

The pH was measured in the supernatants of the previous assays which were about 5.0. Then, the pH of the salt solutions was adjusted to 3.0 and 8.0. A 24 h assay was performed to each salt with a CGC concentration of 15 g L^{-1}. The sampling was performed at 1, 2, 4, 22 and 24 h of the assay. Samples of the salt solutions were also collected.

2.4.2. Nitrogen CGC Particles Solubility and Swelling Capacity

The solubility and swelling capacity of CGC particles in water and in the nitrogen salts was evaluated by performing a 2 h assay with 15 g L^{-1} of CGC. After the end of the assay the particles were collected and placed in a filter to take the excess water from them. They were then weighted and dried in a 70 ºC oven for 24 h. After drying they were weighted again. The solubility and swelling of the particles was calculated according to equations 1 and 2, respectively.

$$Solubility = \frac{CGC\ particles\ before\ drying - CGC\ particles\ after\ drying}{total\ volume\ of\ the\ solution} \qquad Eq.\ 1$$

$$Swelling = \frac{CGC\ particles\ before\ drying - CGC\ weighted\ for\ the\ assay}{CGC\ weighted\ for\ the\ assay} * 100 \qquad Eq.\ 2$$

2.4.3. Nitrogen CGC Particles Production for Release Tests

Further nitrogen CGC particles formation were performed by resuspending CGC in an ammonium nitrate solution for 2 hours. These were the chosen salt and time assay since they resulted in the best results of adsorption of nitrogen salts by the CGC particles. This process consisted in adding 15 g L^{-1} of the milled CGC particles in the solution of ammonium nitrate with gentle magnetic stirring for 2 h. The control of the magnetic stirring was an important aspect to make sure that the particles circulated in the solution (that did not accumulate in the bottom of the cup) in order to maximize the absorbance capacity of the particles. It was also important to make sure that the magnetic stirring was not to vigorous in order to prevent the collision between the particles and their disruption.

After the ending of the assay, the magnetic stirring was stopped, and the particles deposited in the bottom of the cup, which made it easier to separate the particles from the rest of the solution. The particles were collected and placed in a filter with a 47 mm pore (LLG-Glass microfibre filter, binder free) to remove the excess solution present between the particles, and then were placed and dispersed in a petri dish to be dried in a stove at 70 ºC overnight. After dried, the particles were weighed. About 38 g of CGC particles were produced. The produced particles were sifted in molecular sieves to see the pore of the particles. Tree molecular sieves with different pores dimensions were used. The first used was the smallest, and at last was the largest. The particles were separated by their dimensions. The molecular sieves used were with aperture 1.40 mm and mesh 14 (serial number 382205); aperture 2.00 mm and mesh 10 (serial number 387677); aperture 2.36 mm and mesh 8 (serial number 387678), (Laboratory test sieve, ASTME 11, Endecotts LTD., London England, Patent number 667924). Major of the produced particles had an aperture superior than 2.36 mm, about 31.14 g of the 38.32 g total produced.

2.5. Nitrogen CGC Particles Analysis

2.5.1. SKALAR Analysis

SKALAR (Skalar SAN^{++} System) analysis were performed to see the ammonium and nitrate content in the supernatant of the samples collected over time of the different assays. Samples of the salt solution (point zero), time zero (t0), 24 and 48 h were analysed firstly for all the assays performed for the different salts and CGC concentrations, except for urea assays. There was the need to dilute the solutions to reach final concentrations between 0.004 and 0.02 g L^{-1}. The dilution factor was considered in nitrate and ammonium concentration obtained.

2.5.2. Total nitrogen kit

For urea assays analysis, it was used Total nitrogen (TNb) kits LCK 338 (Laton® Hach Lange).

The total nitrogen kit gives the total nitrogen concentration in mg L^{-1} by direct spectrophotometer reading, with a detection limit between 20-100 mg L^{-1}. The concentration of each reagent is unknown. 200 µL of sample is transferred to the tube and 2.3 mL of solution A (sodium hydroxide) and one "pill" of reagent B (dipotassium peroxo disulphate, sodium tetraborate and metaborate sodium) are added, and the tube is closed heated at 100 ºC for 40 min in a HT200S block heater (Hach Lange). After, sample tubes were cooled until reach 20 to 18 ºC, and one capsule of reagent C (sodium azide and sodium sulfite coated in a plastic capsule) was added and the solution shaken until the capsule content was released and dissolved in the solution. Then 500 µL of the sample solution was transferred slowly to the sample flask (60 % sulphuric acid and 33 % phosphoric acid), then 200 µL of reagent D (2-propanol) was slowly added to the flask, shaken and let at repose for 15 min before reading in spectrophotometer (DR 2800 Hach Lange) at 345 nm. The sample flask has a bar code that gives the direct total nitrogen concentration in mg L^{-1}.

2.5.3. Elemental analysis

Elementary analysis was performed to measure the percentage of nitrogen present in the pellet of the samples collected for ammonium nitrate assays and milled CGC using an elemental Analyzer Thermo Finnigan-CE Instruments (Italy), model Flash EA 1112 CHNS, performed in the chemical analysis laboratory from the Chemical Department of the FCT/UNL.This analysis was performed for the nitrogen CGC particles and the original milled CGC, using 3 mg samples. This analysis was performed to see what time of contact between the CGC particles and the ammonium nitrate solution had a major absorbance capacity.

2.6. Nitrogen FucoPol Particles

A 10 g L^{-1} FucoPol solution was used to produce FucoPol particles with the ammonium nitrate encapsulated in them, using a 3.33 g L^{-1} iron (III) chloride hexahydrate solution (Sigma-Aldrich).

Different materials were used to produce particles with different sizes. The ideal was to get a bigger size, where the best shaped and size particle were obtained with the plastic Pasteur pipette (3 mL).

The first approach was to produce spherical FucoPol particles with magnetic stirring in 30 mL of iron (III) chloride hexahydrate solution, and letting them settle in solution for half hour, based on Fialho (2017) approach. But it was observed that particles started to deform and aggregate, also absorbing unnecessary iron (III) chloride hexahydrate solution, which then had to be washed with distilled water for several times. So, in a different approach, a minor volume of the iron (III) chloride hexahydrate solution was used without magnetic stirring.

Six drops of FucoPol solution (10 g L^{-1}) were added to a 500 µL of a 3.33 g L^{-1} iron (III) chloride hexahydrate solution, followed by a gentle manual agitation. Six spheres could be produced without starting to aggregate. These spherical particles were then placed in a teflon Petri dishes and dried in an incubator (LAB-line instruments, inc) at 48ºC for 1h30 min. The dried particles acquired a flat shape.

To produce FucoPol particles with the ammonium nitrate encapsulated, tree different concentrations of ammonium nitrate were tested, 15, 30 and 60 g L^{-1}. A 10 g L^{-1} FucoPol solution was used, and the salt corresponded to each concentration was added directly to the FucoPol solution. The same method, as described previously, was used to produce the nitrogen loaded particles. For the ammonium nitrate concentration of 60 g L^{-1} was more difficult to produce the particles because the solution tended to be more viscous and dense. After the spheres were dried, the ones produced with 60 g L^{-1} of ammonium nitrate were the most difficult to take from the petri dish without breaking them. The particles loaded with 15 and 30 g L^{-1} of ammonium nitrate were easier to collect.

2.6.1. SEM Analysis

FucoPol particles with and without nitrogen salts loaded were analysed by scanning electron microscopy (SEM). The sample was placed in the sample holder of the scanning electron microscope (Hitachi Tabletop TM3030Plus, equipped with SE and a BSE detectors) and freeze dried instantaneously. The analysis was carried out at a temperature of -5 ºC, without coating application. SEM analysis was performed at Instituto Superior de Agronomia (ISA-UL).

2.7. Release Tests in Water

2.7.1. Nitrogen CGC Particles

The release profile of nitrogen CGC particles was studied in water. Therefore, 100 mL of deionized water was poured in 2 g of nitrogen CGC particles and that water was collected and replaced by 100 mL of fresh deionized water. Four replicas were made and the water was changed daily for 17 days. This test was performed in a stove (Trade Raypa) under controlled temperature, at 25 ºC without stirring.

Another test was performed at the same time, 40 samples each with two replicas were prepared in sample flasks of 60 mL with 0.1 g of nitrogen CGC particles in 5 mL of water that were incubated at 25 ºC without stirring. In this test each sample was used for each collecting day, without changing the water.

In these two release tests were used the particles with a size superior to 2.36 mm.

Nitrogen CGC particles with size equal to 1.40 mm were also used to test the release profile in water, where 5 mL of distilled water was poured in 0.1 g of these particles, and water was collected and substituted for the same volume for 17 days. Two replicas were prepared.

Conductivity and pH were measured for all the collected samples. Samples were then frozen at -20 ºC for further analysis of ammonia and nitrate concentration by SKALAR.

2.7.2. Nitrogen FucoPol Particles

Release tests in water were performed with FucoPol particles loaded with the 15 and 30 g L^{-1} ammonium nitrate solutions. Five dried particles were placed in a 60 mL sample flask and 20 mL of autoclaved water was poured in them. This water was replaced by new 20 mL every day for 4 days. Two replicas of each particles were performed. A similar test was performed with the original particles in their spherical form that also lasted for 4 days.

Tests were performed in a stove under controlled temperature, at 25 ºC, without stirring. The conductivity and pH of the collected water was measured. The samples were stored at -20ºC for later SKALAR analysis.

2.8. Release Tests in Soil

2.8.1. Nitrogen CGC Particles

Tests in soil were performed in order to evaluate the release pattern of the nitrogen CGC particles and their durability in soil. At the same time it was used a negative control (without fertilizer), and a control containing commercial fertilizer (SIRO) in the form of beads (composition presented in appendix Table A.1).

For the nitrogen CGC particles and commercial fertilizer, 2 cm high of soil (Green Plants universal – cultivation substrate100% natural) was placed in the pot, then 1 g of each sample were spread and covered with 1 cm high of soil (total soil weight was 27 g for all samples). For the negative control, 3 cm high of soil was placed in the pot. Gaze was placed in the bottom of each pot to prevent soil leakage. Each sample had a replica. Finally 30 mL of deionized water were poured into the soil to saturate it and the excess water was drained to a petri dish and the volume measured. The conductivity of these samples was measured before being stored at -20ºC for further nitrogen analysis by SKALAR and total nitrogen (TNb) kit. Every day 10 mL of distilled water were added to the soil, and the volume of collected water measured. The humidity and pH of the soil was also measured before and after adding the water, using a humidity and pH sensor for soils (natcare 4terra, jardin and natura). This assay lasted for 14 days for control and nitrogen CGC particles, the commercial fertilizer assay lasted 13 days, because the soil and water collected had started to release a bad smell indicating microbial activity.

2.8.2. Nitrogen FucoPol Particles

The process and conditions used to study the pattern release of FucoPol particles in soil was the same described above for nitrogen CGC particles. In this one, the nitrogen FucoPol particles used were ten and their weight was measured (15 g L^{-1}: 0.0121 and 0.0116 g; 30 g L^{-1}: 0.220 and 0.0250 g). This assay lasted for 16 days.

2.9. Nitrogen CGC and FucoPol Particles Release Tests *in vivo*

Tests *in vivo* using pea seeds were performed for nitrogen CGC particles, for nitrogen FucoPol particles, commercial fertilizer and a negative control. All tests were realized in duplicate.

Tests were conducted in a plant tray with 14 inserts. Each insert (cell) was filled with 15 g of soil, then two pea seeds were placed distant from each other and buried in the soil. For the negative control, there was only the seeds, for nitrogen CGC particles was weighted 0.5 g, for nitrogen FucoPol particles there was 5 inserts (15 g L^{-1}: 0.0064 and 0.0071 g; 30 g L^{-1} 0.0100 and 0.0125 g for each replica), and for the commercial fertilizer was weighted 0.5 g. The planted pea seeds were weighed (between 0.4-0.5 g for two pea seeds) and buried in the soil, and then the particles were spread on top of the soil, and covered. About 20 mL of distilled water was poured in each sample in order to irrigate the soil, the excess water was collected and their conductivity measured and preserved at -20 ºC for further analysis. The soil was irrigated almost every day according to the water necessity. Germination of some of the seeds started in the fourth day after plantation. Every day, the growing of each plant was measured. After 34 days, the plants were collected from the soil and their roots were cleaned with distilled water to remove the soil on them, and afterwards dried in paper. Before freeze-dry (Scanvac), each plant was weighted and stored in a 60mL sample flask and frozen at -80 ºC in a ultrafreezer (Arctiko). After the plants being freeze-dried they were weighted again to calculate their dry weight.

Another assay was performed with the pea seeds, but using plastic pots for each sample. It was performed a negative control, nitrogen CGC particles (0.7 g), commercial fertilizer (0.7 g), FucoPol particles (7 particles each; 15 g L^{-1}: 0.0156 g; 30 g L^{-1}: 0.0309 g). In each pot, it was putted two pea seeds (weight between 0.4-0.5 g) with some distance between them. Soil was irrigated every day with 4 mL of deionized water, measurements of the growing plants were performed, and the ambient temperature registered. Germination of some of the seeds started in the fourth day after plantation. This assay lasted for 24 days.

Lastly, another assay was performed in the same conditions as before, but using the FucoPol particles loaded in the 15 and 30 g L^{-1} ammonium nitrate solution in the original state as a spherical hydrogel. Seven particles (15 g L^{-1}: 0.3518 g; 30 g L^{-1}: 0.3448 g) were deposited above the grounded two pea seeds (weight between 0.3-0.4 g). For nitrogen CGC particles assay, was

used 0.35 g of particles. This assay lasted for 16 days, seeds started to germinate in the fourth day. Further sample treatment and analysis were the same made for the first assay.

3. Results and Discussion

3.1. Chitin-Glucan Complex (CGC) Characterization

The biopolymer extracted from *Komagataella pastoris* biomass was obtained as a slightly yellow powder. The CGC had glucose and glucosamine contents of 9.44 and 2.12 wt%, respectively, that correspond to a β-glucan:chitin molar ratio of 84:16, which is the same reported by Roca *et al.* (2012) and slightly different from the molar ratio (75:25) described by Farinha *et al.* (2015). The observed differences may be due to the extraction method used by Farinha *et al.* (2015) that was based on the use of NaOH 5 M, while in this study was used NaOH 1 M.

GCC wasn`t totally purified during the extraction since mannose was not completely removed, has shown by the mannose content of 7.97 wt%, which was lower to the one reported by Roca *et al*. (2012) (28 wt%). CGC had a low inorganic salts content of 2.7 wt%.

3.2. Chitin-glucan Complex (CGC) Particles

3.2.1. Adsorption Assays

Nitrogen CGC particles were produced by testing the adsorption capacity in four different saturated salt solutions (ammonium sulphate, potassium nitrate, ammonium nitrate and urea), taking into consideration the different ionic charges of each salt. Tests were performed for 48 h with magnetic stirring.

Different content of CGC particles (15, 20, 25 and 30 g L^{-1}) were tested in the different salt saturated solutions. Samples taken throughout the tests were analyzed regarding the ammonium and/or nitrate content. The CGC particles absorption capacity was calculated based on the difference between the initial concentration of each ion in solution (point zero) and the ammonium and/or nitrate concentration in the samples collected at different times (t0 (right after the polymer addition), 1 h, 5 h, 30 h, 24 h and 48 h). The blank analysis, performed in deionized water with no salt added, allowed to verify that the polymer suffered no degradation or reaction during the 48 h assay. In Table 3.1 is described the ammonium and nitrate solution content for all the tests performed for the point zero, t0, 24 h and 48 h samples.

For the ammonium sulphate saturated solution, the greater ammonium concentration decrease (5.21 and 13.97 g L^{-1}) was observed for the assays with 15 and 20 g L^{-1} of CGC particles in a period of 48 and 24 h, respectively. However, for the 20 g L^{-1} CGC particles solution between 24 and 48 h, the ammonium concentration in the supernatant increased from 89.67 to 95.18 g L^{-1}, probably due to a desorption process. The same behavior was observed in the solution containing 25 g L^{-1} of CGC particles, nevertheless within 24 h the ammonium decrease was lower (4.17 g L^{-1}) than in the assay with 20 g L^{-1} CGC particles. For the test with 30 g L^{-1} of CGC particles

the decrease of the ammonium concentration was very low (1.89 g L^{-1}). Therefore, the run with 20 g L^{-1} showed the best adsorption values for ammonium sulphate in a short period of time (24 h).

Concerning the tests performed with ammonium nitrate saturated solution, it is possible to observe a much higher ammonium adsorption (37.69-61.16 g L^{-1}) than for any of the CGC concentrations tested in the runs with ammonium sulphate (Table 3.1).

Table 3.1. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations in a 48 h adsorption assay for 15, 20, 25 and 30 g L^{-1} of CGC particles in saturated ammonium sulphate, ammonium nitrate, potassium nitrate solutions and blank solutions.

Salt	CGC (g L^{-1})	Sample time (h) / Ammonium (NH_4^+) (g L^{-1})				Total ammonium uptake (g L^{-1})	
		Saturated solution	t0	24	48	24	48
Blank	15	0.00	0.00	0.00	0.00	-	-
	20	0.00	0.00	0.00	0.00	-	-
	25	0.00	0.00	0.00	0.00	-	-
	30	0.00	0.00	0.00	0.00	-	-
Ammonium Nitrate	15	119.18	69.75	64.94	58.02	54.24	61.16
	20	120.44	73.66	74.60	67.15	45.84	53.29
	25	123.81	74.04	85.06	70.99	38.75	52.82
	30	125.43	78.81	82.58	87.74	42.85	37.69
Ammonium Sulphate	15	98.96	97.66	97.94	93.75	1.02	5.21
	20	103.66	96.32	89.70	95.18	13.96	8.48
	25	100.02	101.91	95.85	98.86	4.17	1.17
	30	98.14	96.30	96.99	96.26	1.84	1.89
Salt	**CGC (g L^{-1})**	**Sample time (h) / Nitrate (NO_3^-) (g L^{-1})**				**Total nitrate uptake (g L^{-1})**	
		Saturated solution	**t0**	**24**	**48**	**24**	**48**
Ammonium Nitrate	15	104.11	72.67	71.89	68.43	32.22	35.68
	20	102.99	65.31	63.29	55.27	39.70	47.71
	25	112.40	56.88	61.42	47.99	50.98	64.40
	30	109.44	52.22	52.12	50.04	57.32	59.40
Potassium Nitrate	15	35.38	15.07	12.32	11.34	23.06	24.04
	20	36.01	10.74	9.63	12.02	26.31	23.99
	25	35.75	8.52	7.35	7.05	28.40	28.70
	30	38.79	11.97	6.29	7.59	32.5	31.20

The ammonium nitrate salt was chosen due to the possibility of increasing CGC particles in nitrogen with the adsorption of both ammonium and nitrate. Therefore, within the 48 h, ammonium adsorption values decreased with the increase in CGC particles content (Table 3.1), being similar (52.82-61.16 g L^{-1}) for CGC concentrations of 15, 20 and 25 g L^{-1} and lower for the concentration of 30 g L^{-1} (37.69 g L^{-1}). Regarding nitrate uptake, it was greater for higher concentrations of CGC. It is noteworthy that most of the ammonium and nitrate uptake occurred in the first minutes of the run (t0; Table 3.1), which may be related with the lower solution pH (~4.15), since for more acidic pH value the chitin amino groups are protonated and to maintain neutrality negative counter-ions (NO_3^-) are necessary (Longhinotti *et al.*, 1998; Iqbal *et al.*, 2011).

On the other hand, at this pH, glucose is more negatively charged leading to a need of positive counter-ions (NH_4^+).

In the potassium nitrate ammonium assay the nitrate uptake was similar, within 23.99 and 31.20 g L^{-1}, for all the CGC particle contents tested. Furthermore, most of the adsorption occurred in the beginning of the test (t0; Table 3.1).

For the urea samples, the nitrogen concentration was determined by the total nitrogen kit (Table 3.2). Nitrogen uptake was close to zero for all the CGC content values studied. Such behavior may be related with the neutral charge of urea that may not have had any interaction with the polymer. According to Hussain *et al.* (2012) in a FTIR analysis to chitosan microspheres with urea encapsulated, it was verified that there was no significant interaction between urea and chitosan molecules.

Table 3.2. Total nitrogen in a 48 h adsorption assay with 15, 20, 25 and 30 g L^{-1} of CGC in an urea saturated solution.

Salt	CGC (g L^{-1})	Sample time (h) / Total Nitrogen (g L^{-1})			
		Zero point	t0	24	48
Urea	15	0.06	0.05	0.06	0.06
	20	0.07	0.07	0.07	0.06
	25	0.07	0.07	0.07	0.06
	30	0.06	0.07	0.06	0.07

Overall, the ammonium and nitrate uptake showed better results at CGC 15 g L^{-1}. At this concentration, the particles collisions during the assay, caused by the magnetic stirring, was less significant. On the other hand, it was observed that at the highest CGC content values tested, there was a tendency of the particles to sediment and accumulate at the bottom of the beaker, even with the constant magnetic stirring. This fact may have contributed to the lower polymer particle adsorption values obtained at 25 and 30 g L^{-1}. In addition, the longer the assay time was, more collisions occurred between the particles leading to their disintegration. For these reasons, the CGC content of 15 gL^{-1} and a 24 h time period were chosen for further work.

3.2.2. Effect of pH on CGC Particles Adsorption

The impact of different pH values (3.0 and 8.0) on CGC adsorption of the different salts was evaluated in a 24 h assay (Figure 3.1 a and b).

It was verified in the pH 3.0 assay (Figure 3.1 a), that after the polymer addition there was a pH increase, being more significant for the potassium nitrate and ammonium nitrate (about pH 5.5). It was noted that after the 4 h of the assay, the pH tended to stabilize until the 24 h. In acidic conditions, the cationic amino groups from CGC are protonated in the presence of H^+ and are electrostatically attracted by the NO_3^- ions from the dissociated potassium nitrate and ammonium

nitrate, leading to an increase in the solution pH in the first 4h assay, and until the 24 h the ions reached the equilibrium not verifying further pH variations (Wong *et al.*, 2004).

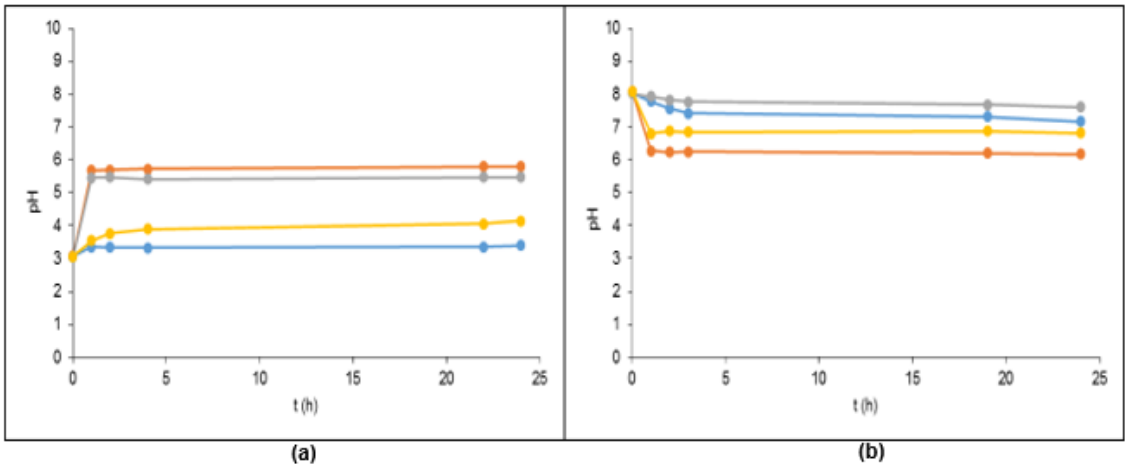

Figure 3.1. pH variation in a 24 h period for ammonium sulfate (●), potassium nitrate (●), ammonium nitrate (●) and urea (●) solutions, at initial pH 3.0 **(a)** and 8.0 **(b)**, for 15 g L^{-1} of CGC.

In the pH 8.0 assay (Figure 3.1 b), in general there was a decrease in the pH value after polymer's addition, and after 3 h, the pH was practically constant until the 24 h. It was noted that for the urea (●) and potassium nitrate (●) solutions, after 1 h assay the pH decrease was more significant (urea: from pH 8.08 to 6.79; potassium nitrate from pH 8.08 to 6.28). According to Longhinotti *et al.* (1998) higher pH, the lowest protonated are the amino group from chitin, resulting in a lower interaction between these groups and the anionic groups from the ammonium nitrate and ammonium sulphate. In aqueous solution urea dissociates into CNO^- and NH_4^+, but at neutral pH this dissociation is slower than at an alkaline medium (the activation energy was lower) (Alexandrova and Jorgensen, 2007). It was deduced that in the alkaline solution the urea dissociated more rapidly, and the ions NH_4^+ competed with the deproteined amino groups from chitin for the HO^- ions in solution, and the same could had happened with the K^+ ions from potassium nitrate, leading to a decrease in the solution pH in the first hour.

Supernatants of the samples taken from the runs at initial pH 3.0 and 8.0 were analyzed for their content in ammonium and nitrate. However, these results were not conclusive (data not shown), since there were no significant changes in the ammonium and nitrate concentration. Therefore, it was decided to evaluate the nitrogen present in the pellets (nitrogen CGC particles) of the samples taken in the pH 3.0, 5.0 and 8.0, taken at 24 h, by elemental analysis (Table 3.3).

By comparing the CGC sample with the remaining in terms of nitrogen composition, it was verified that there was in fact nitrogen adsorption by the polymer when in contact with the ammonium nitrate saturated solution for all the pH values tested (Table 3.3), contradicting the ammonium and nitrate content results of the supernatants (data not shown). The pH 5.0 assay

was the one that demonstrated a greater nitrogen adsorption by the polymer in comparison with the remaining assays, although there were no significant differences between them.

Table 3.3. Nitrogen, carbon, hydrogen and sulphur content (%) for milled CGC and for 15 g L^{-1} CGC particles in ammonium nitrate, for samples 24 h of the assay at different pH values of 3.0, 5.0 and 8.0.

%	CGC	pH 3.0	pH 5.0	pH 8.0
Nitrogen	6.03	29.54	30.63	24.07
Carbon	45.64	9.45	6.75	15.35
Hydrogen	7.26	5.35	4.99	5.50
Sulphur	0.15	0.00	0.00	0.00

Since the difference in nitrogen content in the CGC particles from the different assays with pH variation was not significant, the work was continued with solutions at pH 5.0 (natural pH solution).

Another ammonium nitrate solution at pH 5.0 with 15 g L^{-1} of CGC assay was performed in a 24 h period, to see which time had the best nitrogen adsorption capacity (Table 3.4). It was noted that the highest nitrogen content in the particles occurred within 30 min, 1 h and 2 h after polymer submersion in the saturated solution. Although at 30 min there was more nitrogen uptake in the CGC particles, the 2 h time was chosen to produce the particles, since it was performed tree replicas for this sample, confirming the nitrogen uptake result, that was also satisfying. There was no replicas of the other samples, because the elemental analysis was expensive.

Table 3.4. Nitrogen, carbon, hydrogen and sulphur contents (%) for the 15 g L^{-1} CGC particles in ammonium nitrate in the initial pH 5, in a 24 h assay.

%	CGC	30 min	1 h	2 h	4 h	6 h	24 h
Nitrogen	6.18	31.91	29.89	28.73	27.89	27.70	26.64
Carbon	44.93	7.69	9.59	9.90	10.48	11.10	10.79
Hydrogen	7.61	5.81	4.99	4.94	5.33	4.23	5.23
Sulpher	0.22	0.00	0.00	0.00	0.00	0.00	0.00

The average of nitrogen adsorption at 2 h contact period was of 28.73% (± 0.35 %). Taking into consideration that the nitrogen present in milled CGC was 6.18% the nitrogen CGC particles adsorption capacity at the end of two hours is of 22.55 %.

3.2.3. Chitin-glucan Complex (CGC) Swelling Capacity

CGC particles (15 g L^{-1}) immersed in the different salts and in distilled water for 2 h contact with magnetic stirrer, were collected and dried overnight at 70 ºC. The swelling capacity was calculated as the difference in mass between the wet and dry masses (Table 3.5).

Table 3.5. Solubility (g mL^{-1}) and swelling capacity (%) for 15 g L^{-1} of CGC in distilled water for 2 h.

Sample	Solubility (g mL^{-1})	Swelling (%)
Water	0.04	219.08
Ammonium nitrate	0.02	208.28
Ammonium sulphate	0.01	112.26
Potassium nitrate	0.06	431.47
Urea	0.03	291.83

CGC particles demonstrated a high swelling capacity (219.08 %) for a short period of time (2 h) in water (Table 3.5), which is in accordance with results reported by Farinha *et al.* (2015). CGC particles in ammonium nitrate solution also demonstrated a high swelling capacity (208.28 %), but relatively lower than that of water. According to Li *et al.* (2016) that could be due to the existence of additional ions in solution that could decrease the swelling capacity due to the decrease of the osmotic pressure gradient between the polymer matrix and the solution. The same possibly happened in the ammonium sulphate solution, CGC had the lowest swelling capacity (112.26 %), and in addition was verified the particles were smaller, didn`t swelled as much as with the other salts, possibly due to a higher ion concentration in solution (5.47 M) and the ions SO_4^- and the $[NH_4^+]_2$ are bigger ions, if they were uptake by the polymer, they could had increased the cross-link degree of the matrix decreasing the polymer swelling capacity.

In the potassium nitrate and urea solutions, CGC showed a high swelling capacity (431.47 and 291.83 %, respectively), in the case of potassium nitrate, a lower salt concentration (3.09 M) may have increased the osmotic pressure gradient towards the polymer matrix, and led to higher water penetration into the internal polymer matrix. As the NO_3^- is a small ion, it could had easily entered the polymer network and connected to the positive charged free amino groups, Wong *et al.* (2004) reported a similar phenomenon. In the case of urea, a high swelling capacity may be due to the free water absorption, since previous adsorption tests demonstrated that there was no nitrogen uptake by the polymer in the urea solution, this may be due to the salt neutral charge that did not had interaction with the polymer, having a stronger bond with the water. According to Li *et al.* (2016) a good slow release fertilizer should have good water retention properties to improve the soil irrigation capacity.

This proves that this polymer is suitable for applications that need a good adsorption matrix. Figure 3.2 shows the CGC particles after being immersed in ammonium nitrate saturated solution (Figure 3.2 a) and after being dried (Figure 3.2 b) acquiring a rigid texture.

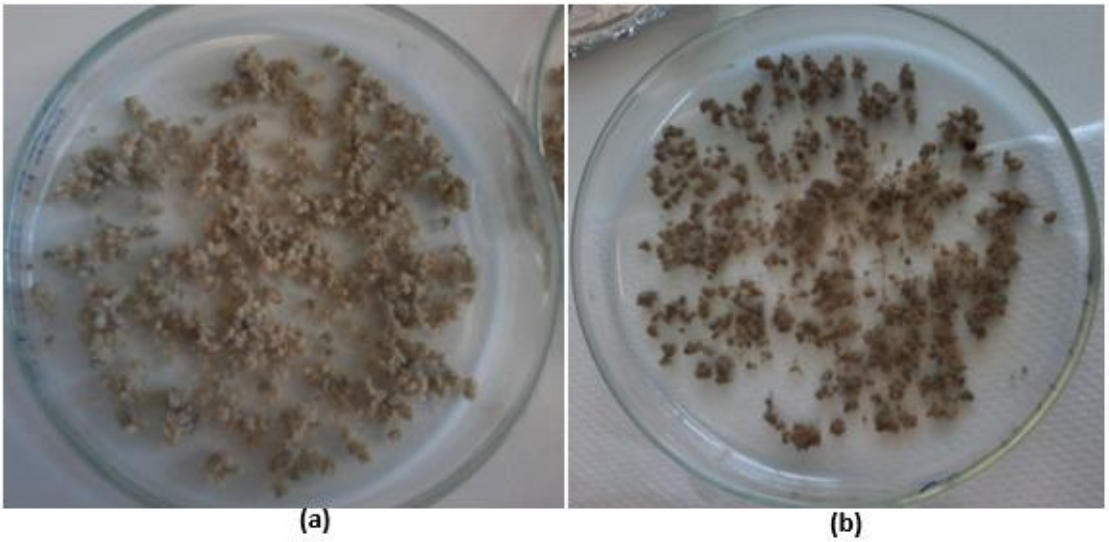

Figure 3.2. (a): 15 g L^{-1} of CGC particles collected after 2 h assay in saturated ammonium nitrate solution; **(b):** CGC particles dried overnight at 70ºC.

3.2.4 Water Release Test with Nitrogen CGC Particles

Ammonium and nitrate release from the nitrogen CGC particles with a size of ≥ 2.36 mm were tested in water at 25 ºC using four replicas with sample collecting and changing water every day, during 22 days.

Results showed that the ammonium and nitrate concentration at the beginning of the assay with the first change of water from the particles was of 0.66 and 0.69 g L^{-1}, respectively and dropped very significantly at the third day to 0.024 and 0.025 g L^{-1}, respectively (Figure 3.3 a and b). At the fifth day the nitrate and ammonium content in solution was practically zero. This could mean that all or most of the ammonium and nitrate adsorbed to the polymer particles was release in the beginning of the test (hours). The total N uptake was calculated based in the elemental analyses result (22.55 %) and for 2 g of particles was estimated to have an initial 0.28 g L^{-1} of N. But the initial ammonium and nitrate released into the water was higher (0.66 and 0.69 g L^{-1}, respectively). Since the CGC particles behave differently between them, in terms of their adsorption capacity and nitrogen uptake, the estimated N uptake could not be close to the real initial nitrogen uptake in the particles, and so it wasn't possible to estimate with sure the real initial N uptake in the particles.

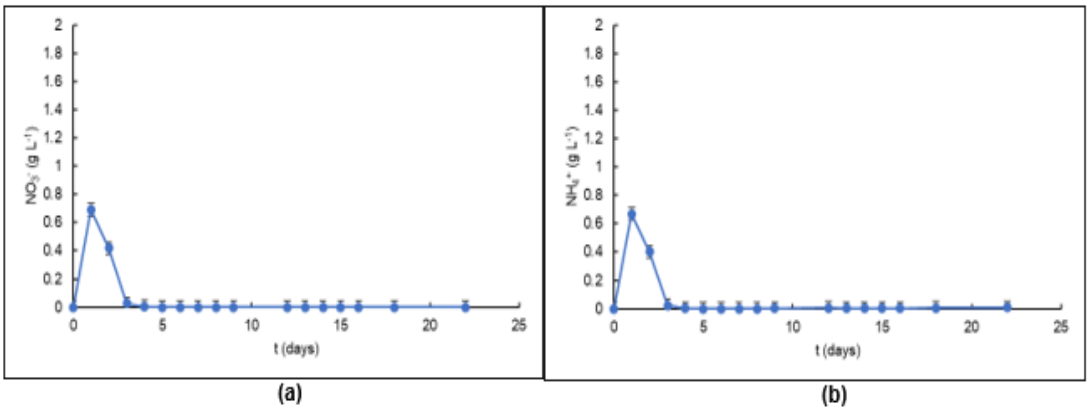

Figure 3.3. (a): Nitrate (NO_3^-) concentration (g L^{-1}) and **(b):** Ammonium (NH_4^+) concentration (g L^{-1}) vs time (days) average of four replicas in water test at 25 ºC with water changes.

The higher release observed in the beginning of the assays (Figure 3.3) could had been an initial burst release as reported by Hussain *et al.* (2012), where a higher urea content from chitosan microspheres led to an increase in the released rate in water due to the concentration gradient differences, releasing by diffusion. It was also reported by Roshanravan *et al.* (2015) and Sempeho *et al.* (2015) by working with urea encapsulated in kaolinite that a great part of the urea was release in water in the first days, since urea is very volatile. It was also reported by Kashyap *et al.* (2015) that a burst release by osmosis can occur when the nutrients are adsorbed to the particles surface, which was also the case of these particles. But to confirm if there was more nitrogen uptaken into the polymer matrix, a longer assay should had been performed. However a water assay is a more “aggressive” environment than soil, it has probably accelerated the diffusion of the ammonium and nitrate ions out of the particles (Melaj and Daraio, 2013).

Another test was performed in the same conditions, but using nitrogen CGC particles with a lower particle size (1.40 mm), to see if there was any difference in the pattern release with the previous assay. The nitrate and ammonium released at the first day was of 1.07 and 1.00 g L^{-1}, respectively, and at the third day dropped to 0.03 g L^{-1} for both (Figure 3.4 a and b). At the fifth day it was practically zero. The estimated initial nitrogen uptake was calculated in 0.014 g L^{-1}, but the released in the first day was higher than that, which could be due to the situation reported above (Figure 3.3). The release pattern was identical to the previous assay, which indicates that possibly the ammonium and nitrate was all or in part release in the first two days (Figure 3.4). The assay lasted 22 days, but the first 8 days samples were analyzed and the rest was not because there was very little traces of ammonium and nitrate in the samples.

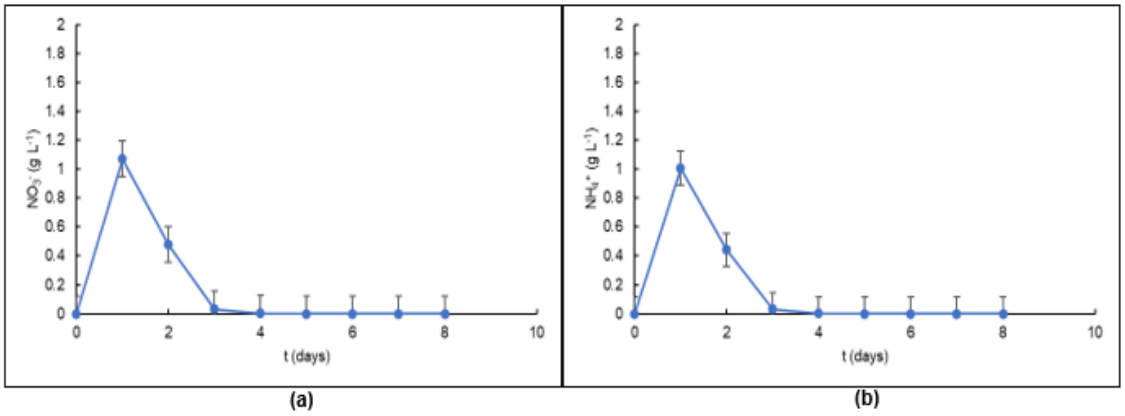

Figure 3.4. (a): Nitrate (NO_3^-) concentration (g L^{-1}) and **(b):** Ammonium (NH_4^+) concentration (g L^{-1}) vs time (days) average of two replicas in water test at 25 ºC with water changes using 1.40 mm particles.

It was also performed a release test without changing the water for ≥ 2.36 mm particles (Figure 3.5 a and b). Two replicas were made to use in each day (average represented in Figure 3.5), to see the release pattern without changing the water. At the first day the nitrate and ammonium release content was 1.59 and 1.50 g L^{-1}, respectively, in the second day, and at the fourth day it seems that there was a slight drop in the fourth day to 1.64 and 1.55 g L^{-1} (pH drop from 4.73 to 4.68 registered), respectively, that could be due to a desorption process. With the adsorption interaction between the nitrogen ions and the polymer the pH slightly dropped (4.76 to 4.71) and during the assay it oscillated between 4.79 to 4.64 that may be due to adsorption and desorption phenomenon. The initial nitrogen uptake from the particles was estimated to 0.014 g L^{-1}, and as happened to the previous assays was lower than the ammonium and nitrate content released. (Figure 3.5).

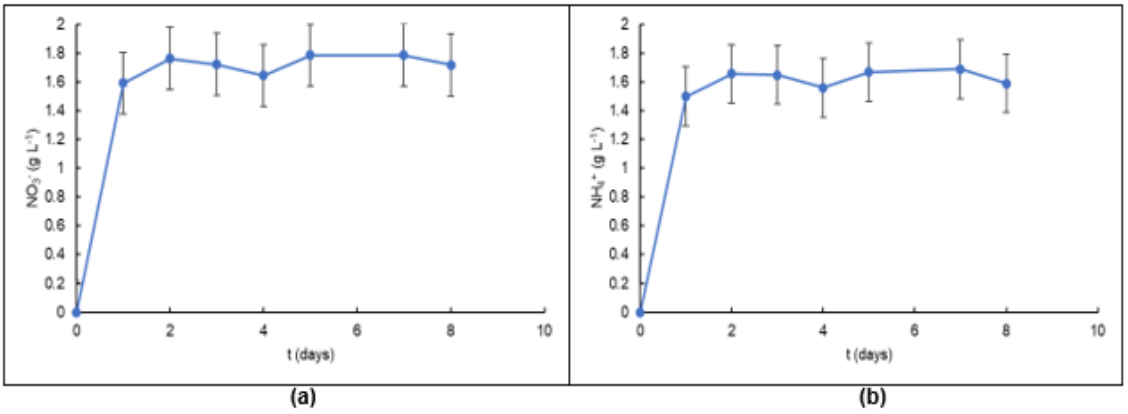

Figure 3.5. (a): Nitrate (NO_3^-) concentration (g L^{-1}) and **(b):** Ammonium (NH_4^+) concentration (g L^{-1}) vs time (days) average of two replicas in water test at 25 ºC without water changes using ≥ 2.36 mm particles.

In general, it is not certain if the ammonium and nitrate content that was adsorbed in the polymer particles was all or partially released in the first contacts with water. To confirm this, a

longer release water test should be performed, and periodical analysis to the nitrogen content of the particles should also be performed. Also, these tests could be performed taking into account the pH effect, to understand the polymer and nitrogen ions interactions during the assays.

According to Hussain *et al.* (2012) with a higher urea content in the chitosan microspheres (1.5 g) about 40 % of the urea content was released in the first day and the rest released in seven days, which is what could be happening to these particles in water. A higher chitosan content would confer resistance to the microsphere wall and slower the urea release (about 10 % released in the first day). It was reported by Trinh *et al.* (2014) that a commercial fertilizer Agrium® urea coated was tested in water, and showed a satisfying release profile for a controlled released, where in the first five days had a 3.19 % release, and in 82 days 99.1 % of the nitrogen content was released, which accomplished the European Standardization Committee (CEN) that proposed that at 25 ºC, it shouldn`t release no more than 15 % of the total nutrient content in 24 h and no more than 75 % in 28 days (Trenkel, 2010; Roshanravan *et al.*, 2015).

3.2.5. Soil Release Tests with Nitrogen Chitin-glucan Complex (CGC) Particles

The release pattern of nitrogen CGC particles in soil were also evaluated. Particles were buried at 1 cm depth in the soil and watered daily. The excess water was collected to analyze the ammonium and nitrate content. A negative control and a commercial fertilizer group were tested for comparison. All tests were performed in duplicate and averages are presented (Figure 3.6 a, b and c).

The control only had soil, and the concentration of ammonium and nitrate showed to be very low in the soil (< 0.002 g L^{-1} for ammonium and < 0.02 g L^{-1} for nitrate) (Figure 3.6 a and b).

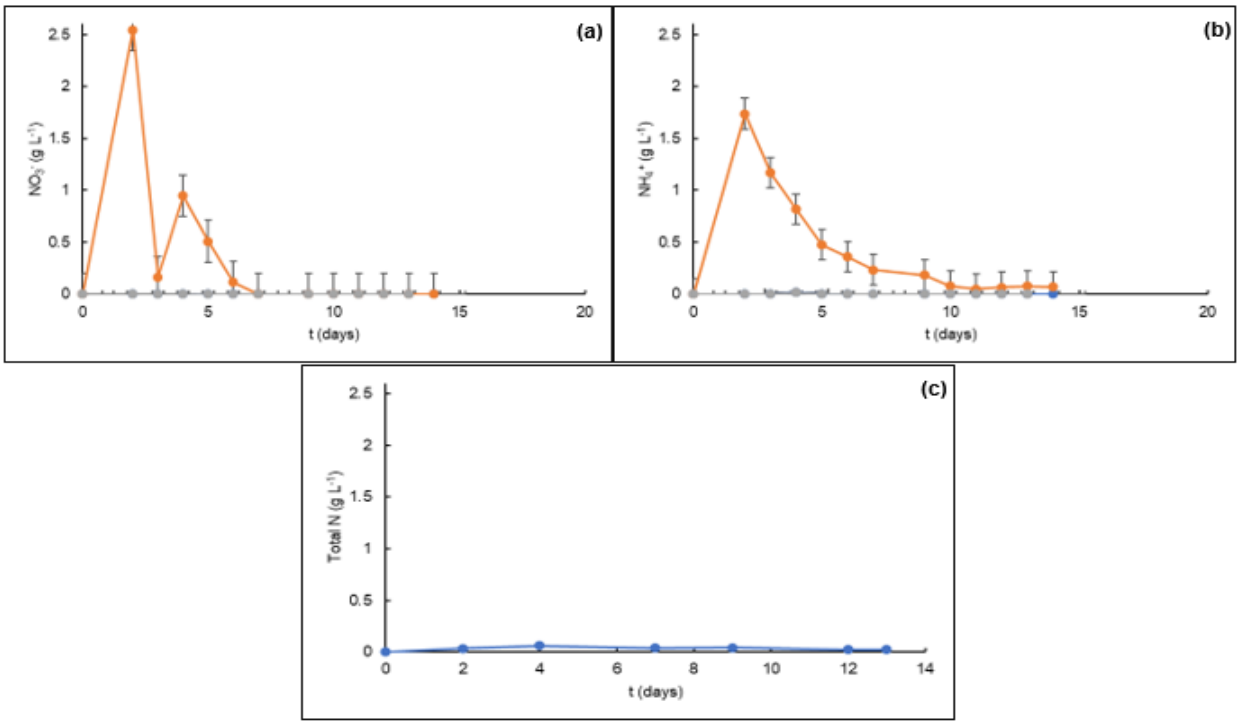

Figure 3.6. (a): Nitrate (NO_3^-) concentration (g L^{-1}) and **(b):** Ammonium (NH_4^+) concentration (g L^{-1}) vs time (days) average of two replicas in soil test for a control (●), for nitrogen CGC particles (●) and for commercial fertilizer (●). **(c):** Total nitrogen (g L^{-1}) from some of the commercial fertilizer samples (●).

Nitrate and ammonium release patterns from nitrogen CGC particles were also evaluated. In the second day about 2.54 g L^{-1} of nitrate was release, and for the rest of the days their concentration was lower (< 0.94 g L^{-1}) and at the ninth day was practically zero (Figure 3.6 a).

Regarding the ammonium release from nitrogen CGC particles (Figure 3.6 b) at the second day there was about 1.73 g L^{-1}, but for the next 5 days the ammonium release was decreasing linearly. At the 10th day the release of ammonium was 0.08 g L^{-1} and maintained relatively linear until the end of the assay. Despite the released amount was superior to the water release tests (section 3.2.4), was also verified the initial burst release, in the next days the release was decreasing linearly. The estimated initial nitrogen amount of the particles was of 0.14 g L^{-1}, but the released content was superior. It is believed that the real nitrogen content presented in the particles was higher. Since there was still ammonium being released in a relatively constant amount, a longer release test should had been performed, to confirm if there was nitrogen linked inside the polymer matrix, that could had not been released yet.

For the commercial fertilizer the nitrate presence was residual as expected since it is not part of the chemical composition of this fertilizer, and the ammonium content was in general lower than 0.015 g L^{-1} (Figure 3.6 a and b). The estimated nitrogen content was 0.18 g L^{-1}. Some of the samples from the commercial fertilizer assay were analyzed for nitrogen quantification using the total nitrogen kit, since 16.8 %of the 18 % nitrogen content is from urea (Figure 3.6 c). The nitrogen content in the second day was 0.035 g L^{-1} and in the fourth day was 0.058 g L^{-1}, this

increase may be due to leaching. Until the last day assay the nitrogen content oscillated between 0.039 and 0.022 g L^{-1}. At the end of the assay, these particles were intact, the nitrogen content could had been due to the degradation of one of the beads, or by the soil leaching, since there is no data on nitrogen content of soil.

Moisture is one of the soil properties that affect the release behavior of the nutrients. It also allows to study the ammonium volatilization (Timilsena *et al.*, 2014; Roshanravan *et al.*, 2015).

The soil humidity was monitored before and after adding the 10 mL water with a humidity sensor for soil. The humidity was presented in a scale of 1 to 4, being 1 the lowest humidity and 4 the highest humidity. In the negative control the humidity after adding the water was in general between 2 and 4 for both replicas, and did not drop much after a 24h period, staying in the range of 2 and 3. The tests with nitrogen CGC particles the humidity after adding the water was between 3 and 4, and in a 24 h period remained between 2 and 3. The humidity of the commercial fertilizer group after adding the water was between 2 and 3, and in the 24 h period the humidity retained was within 1-3. It was important to maintain the soil with humidity so that it could be possible to collect the water to analyze its ammonium and nitrate content. In the nitrogen CGC particles it was observed that in comparison with the commercial fertilizer it maintained a higher soil humidity during a 24 h period, which may be due to its swelling capacity to retain water molecules.

The water volume collected during the assay for all samples was very low (1-3 mL) in the first day, because the soil was very dry and the first 30 mL added were absorbed by the soil (Figure 3.7). In the second day, a higher quantity of water was possible to collect, since the soil was saturated with water. From the 3rd day ahead most of the water added (10 mL) was collected, demonstrating that the soil was well hydrated. The volume of collected water was relatively similar for all the samples, despite between the 5th and 10th days the volume collected for the nitrogen CGC particles group was lower, that may be due to the water retention in the particles.

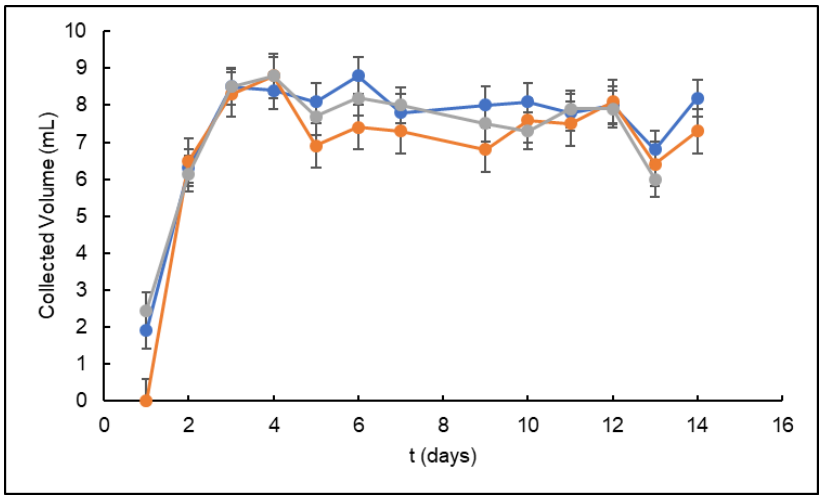

Figure 3.7. Volume (mL) of water collected after the addition of 10mL of distilled water for the control (●), CGC (●) and commercial fertilizer (●) groups.

The soil release tests with nitrogen CGC particles demonstrated that the nutrients release was slower than in water, taking five days to drop to lower values of ammonium content from the particles. It was not possible to deduce if all the ammonium and nitrate content had been released. It was not possible to distinguish the nitrogen CGC particles from the soil, possibly because most of them had disintegrated, may be due to the microorganism's growth in the soil, since it was not sterilized.

The release profile of the control and the commercial fertilizer was similar, which could mean that the commercial fertilizer did not release at least a significant amount of ammonium or nitrogen in the 14 days assay. Besides this, at the end of the assay, when removing the soil from the plastic pots, the commercial fertilizer beads were intact.

3.3. FucoPol Characterization

In terms of sugar content the polymer had a content of 33.09 mol% of fucose that was within the range presented by Torres *et al.* (2015) (32-36 mol%), 23.01 mol% of galactose was lower than the range presented in the literature (25-26 mol%), 37.31 mol% of glucose that was slightly higher than the literature (28-37 mol%) and 4.42 mol% of glucuronic acid that was lower than the range presented in the literature (9-10 mol%).

3.4. Nitrogen FucoPol Particles

FucoPol (10 g L^{-1}) was dissolved in ammonium nitrate 15 and 30 g L^{-1} solutions. To form big and spherical particles (Figure 3.8 a) the solution were dropped, using a plastic Pasteur pipette, in an iron (III) chloride hexahydrate solution (3.33 g L^{-1}), which acts as crosslinker to form

hydrogel particles. These particles were dried at 48 ºC for about 2 h, and presented a flat form (Figure 3.8 b).

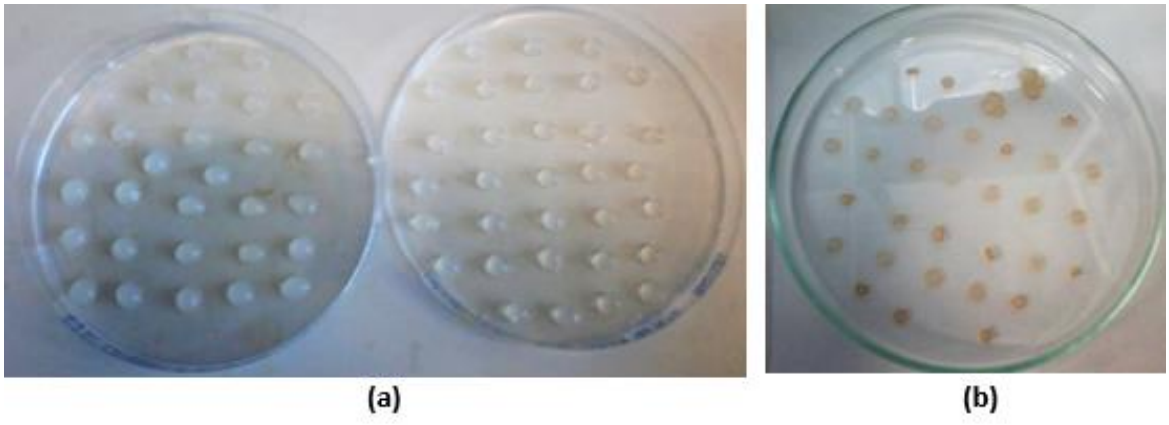

Figure 3.8. (a): Hydrogel particles of 15 (right Petri dish) and 30 g L^{-1} (left Petri dish) of ammonium nitrate in 10 g L^{-1} of FucoPol performed with a plastic Pasteur pipette in a 3.33 g L^{-1} iron (III) chloride hexahydrate solution as crosslinker; **(b):** hydrogel particles dried.

The total nitrogen content in a wet hydrogel particle was determined by the total nitrogen kit, the particles had encapsulated the ammonium nitrate, and as expected the 30 g L^{-1} particle had a higher nitrogen content than the 15 g L^{-1} (Table 3.6).

Table 3.6. Total nitrogen (g L^{-1}) content of FucoPol hydrogel particles with 15 and 30 g L^{-1} of ammonium nitrate encapsulated.

Sample	Total Nitrogen (g L^{-1})
15 g L^{-1} ammonium nitrate in 10 g L^{-1} of FucoPol	0.34
30 g L^{-1} ammonium nitrate in 10 g L^{-1} of FucoPol	0.53

SEM analysis was only possible to do on the hydrogel particles with ammonium nitrate (Figure 3.9 a, b and c). The particles showed a smooth surface and did not showed superficial differences between them. The particles with only FucoPol were not possible to analyze because the surface was translucent, the particles must had degraded because of the heat during the transportation to the place where the analysis was performed.

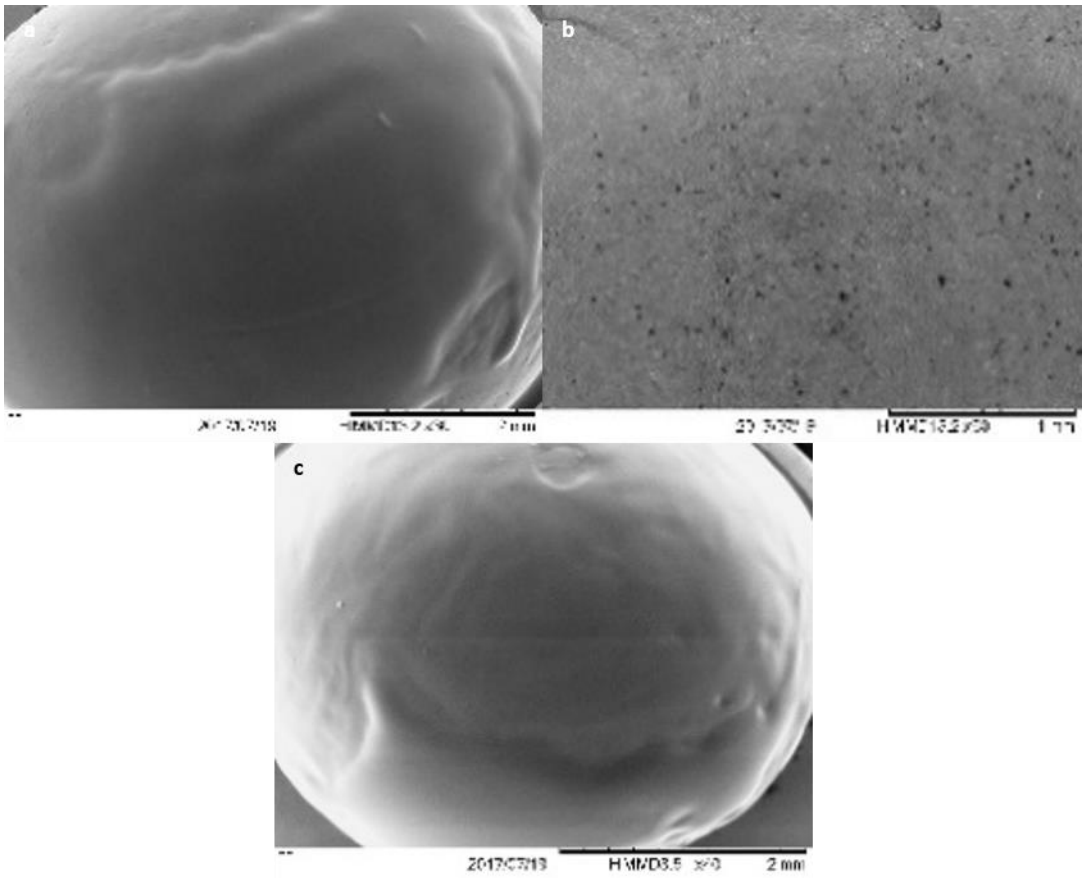

Figure 3.9. SEM analysis of FucoPol hydrogel particles with 15 and 30 g L^{-1} of ammonium nitrate encapsulated, with 30x (15 g L^{-1}) (a), 40x (30 g L^{-1}) (b) and 60x(15 g L^{-1}) (c).

It was decided that both FucoPol particles (15 and 30 g L^{-1} of ammonium nitrate encapsulated) were going to be used for further release tests. Moreover, particles were also produced in a solution of 60 g L^{-1} ammonium nitrate, but it was more difficult to make a hydrogel sphere because the consistency of the polymer was more viscous and dense, and particles had the tendency to agglomerate, so it was not possible to produce many particles at the same time. The dried particle were also more difficult to remove from the Teflon Petri dish, disintegrating, so this concentration was not viable.

3.3.1. Water Release Test with Nitrogen FucoPol Particles

Nitrogen FucoPol dried and hydrogel particles with ammonium nitrate encapsulated, in solutions with 15 and 30 g L^{-1}, were tested in water at 25 ºC, with changes of water every day and sample collecting. Tests were performed in duplicate and averages are presented in Figure 3.10 (a and b).

In the first day the nitrate and ammonium content released was of 0.027 and 0.026 g L^{-1}, respectively for the 15 g L^{-1} and of 0.029 for the 30 g L^{-1} dried particles (Figure 3.10 a and b). In the rest of the days, the released amount content was practically zero.

Also for the hydrogel particles it was verified an initial burst release for the 15 (0.012 for nitrate and 0.013 for ammonium) and for the 30 g L^{-1} (0.023 for nitrate and 0.027 for ammonium), but it could stabilize in the first two days and then would be practically zero, that may be due to the particles started to burst and disintegrating. It was observed that the water change method applied in was aggressive for these spheres, damaging their structure. In the future, to overcome this situation nitrogen FucoPol particles release tests in water should be performed by collecting the particles first and then changing the water. The estimated initial nitrogen content for five hydrogel particles (used in these tests) was 1.72 g L^{-1} for a 15 g L^{-1} particle and 2.64 g L^{-1} for a 30 g L^{-1} particle. The content released was lower than the initial nitrogen estimated, despite overall in the third day assay there was practically no released content from ammonium and nitrate it seems that there was still nitrogen to be released, that could be still linked to the polymer matrix, even after its structure started to disintegrate. In the dried particles, it was observed that the ammonium and nitrate content released was also low (0.027 and 0.026 g L^{-1} for nitrate and ammonium content, respectively for the 15 g L^{-1} and of 0.029 g L^{-1} for nitrate and ammonium for the 30 g L^{-1}), this may be due to losses of salt after the particles were dried, since they acquired a flat shape that could jeopardize the salt encapsulation. It was reported by Jammongkan and Kaewpirom (2010) that the chitosan hydrogels had an initial burst release that tended to stabilize, that could be due to the high amount of potassium encapsulated in hydrogel, due to the osmosis gradient difference.

Like it was seen before for the CGC particles, the water is a more aggressive environment than soil, which could be accelerating the salts diffusion out of the particles.

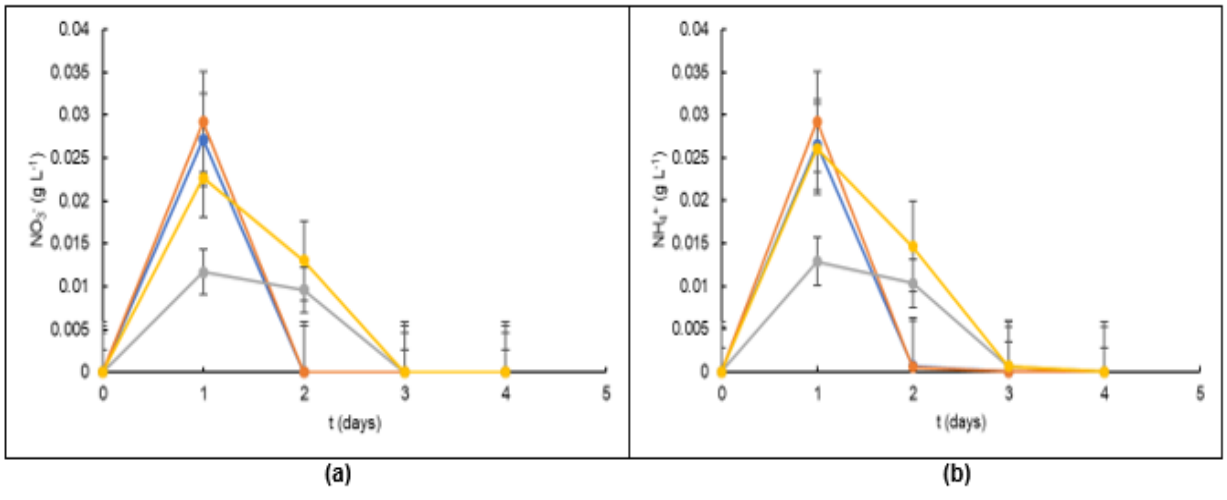

Figure 3.10. (a): Nitrate (NO_3^-) and **(b):** ammonium (NH_4^+) concentration (g L^{-1}) vs time (days) in water test at 25ºC with water changes every day, for the dried particles of 15 g L^{-1} ammonium nitrate encapsulated in FucoPol (●), 30 g L^{-1} dried particles (●), hydrogel particles 15 g L^{-1} (●) and hydrogel particles 30 g L^{-1} (●).

3.3.2. Soil Release Tests with Nitrogen FucoPol Particles

Release tests in soil were also performed for dry nitrogen FucoPol particles, with two replicas where averages are presented in Figure 3.11 (a, b and c). This assay lasted 16 days.

The negative control group (Figure 3.11 a and b) showed an ammonium and nitrate concentration near to zero.

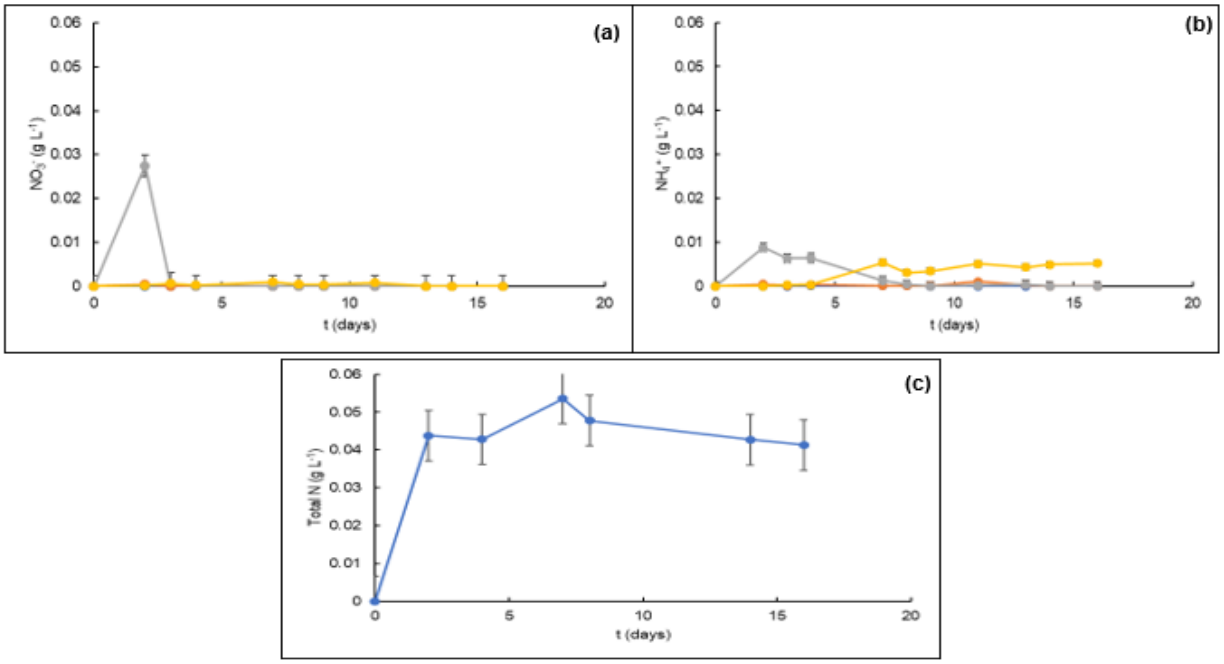

Figure 3.11. **(a):** Nitrate (NO_3^-) concentration (g L^{-1}) and **(b):** Ammonium (NH_4^+) concentration (g L^{-1}) vs time (days) average of two replicas in soil test for a control (●), for ammonium nitrate 15 g L^{-1} in FucoPol dried particles (●), for ammonium nitrate 30 g L^{-1} in FucoPol dried particles (●) and for commercial fertilizer (●). **(c):** Total nitrogen (g L^{-1}) from some of the commercial fertilizer samples (●).

In the FucoPol 15 g L^{-1} particles, the nitrate and ammonium concentration present in the water collected was very low, even though the higher value released (0.0005 g L^{-1} for both nitrate and ammonium) were attained at the second day, in contrast with the water assay where at the first day 0.027 and 0.026 g L^{-1} of nitrate and ammonium were released.

In the FucoPol 30 g L^{-1} particles the nitrate and ammonium content released in the second day was 0.03 and 0.012 g L^{-1}, respectively, while in water tests in the first day was 0.029 g L^{-1} for both nitrate and ammonium. In this assay it was used the double of particles used in water tests, and the estimated nitrogen content was 3.43 and 5.28 g L^{-1} for 15 and 30 g L^{-1} particles, respectively. The ammonium and nitrate ions could be linked in the polymer matrix and was not released or part of the salt was lost during the particles drying process. It was reported by Jammongkan and Kaewpirom (2010) that in soil tests the chitosan hydrogel CRF with potassium nitrate encapsulated suffered an initial burst release and tended to stabilize in a period of 2 to 6 days.

For the commercial fertilizer group, the nitrate presence was residual as expected, and ammonium presence was low (≤ 0.0052 g L^{-1}). Some of the samples from the commercial fertilizer assay were analyzed for total nitrogen, the nitrogen content was around 0.054 g L^{-1} (Figure 3.11 c).

The soil humidity was also measured across the runs. After the water addition, the humidity in negative control was between 3 and 4, and in a 24 h period it was within 1 to 3. And the FucoPol 15 g L^{-1} particles humidity in soil was similar to the negative control, after the water addition was between 2 to 4, and in a 24h period was between 1 and 3.

For the FucoPol 30 g L^{-1} group the humidity after the water addition was between 3 and 4, a good water content in soil, however after 24 h the humidity was within 0 to 3, indicating that soil was not well irrigated. Also for commercial fertilizer assays the humidity after 24 h of water addition was between 0 and 3 and right after water addition soil humidity was between 2 and 3. Overall the humidity was low after a 24 h period, indicating that there probably was water evaporation from the soil, that could had led to a nitrogen volatilization from the FucoPol particles, which could explain the low ammonium and nitrate content.

As for the nitrogen CGC particle tests, the collected volume for the samples was relatively similar (Figure 3.12). As it happened in the CGC assay in the first day very low or no water was possible to collect.

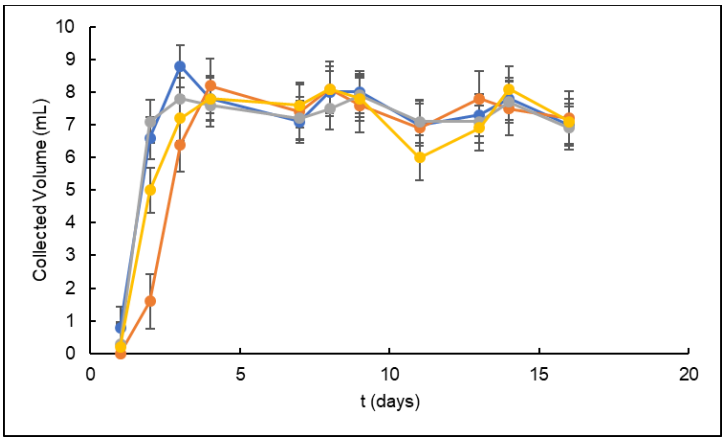

Figure 3.12. Volume (mL) of water collected after the addition of 10mL of distilled water for the (●), FucoPol 15 g L^{-1} (●), FucoPol 30 g L^{-1} (●) and commercial fertilizer (●) groups.

Overall, the release tests in soil for the nitrogen CGC particles demonstrated to be slower, despite the initial burst release. According to Jamnongkan and Kaewpirom (2010) the diffusion in soil is more difficult than in water, expecting that the nutrient release was slower. In the nitrogen FucoPol particles release tests, the released content was low, possibly occurred nitrogen volatilization, since the soil humidity was lower after a 24 h period (0-3).

The manufacturer information about the nutrients release time of commercial fertilizer beads was that it is for a six months release period. The beads consistence was rigid, the assay time was not long enough to see the beads degradation performance. The nitrogen FucoPol particles were not found in the end of the assay, they may have been degraded by microbial action, since the soil was not sterilized.

3.4. *in vivo* Release Tests with Nitrogen CGC and FucoPol Particles

3.4.1. Plant Growth

Using a plant tray with twelve spaces, the nitrogen CGC and FucoPol particles were tested for germination and growth of pea seeds. A control (just with seeds) and the commercial fertilizer were also tested. This assay lasted 34 days (Figure 3.13 a). Another assay with the duration of 24 days was also performed in plastic pots at the same conditions (Figure 3.13 b).

For the control group (Figure 3.13 a), all the seeds were succeeded in germinating. Germination started in the fourth day of plantation and they growth very fast in the first days, continuing to growth until the end of the assay but slower (12.3 cm). In the plastic pot test (Figure 3.13 b) only one of the two seeds germinated and grew 16.5 cm in 24 days, this may be due to the more availability of nutrients in the soil, since the other seed did not germinate.

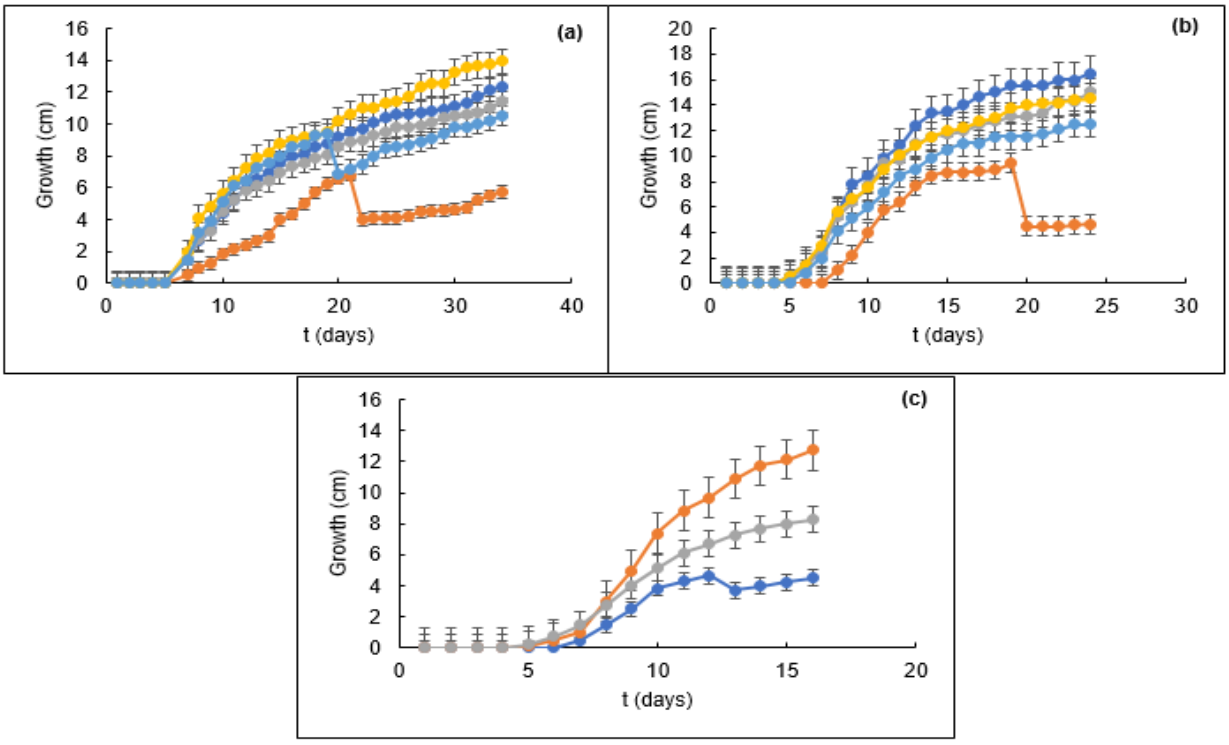

Figure 3.13. (a): Pea growth in a 34 day assay in a tray and **(b)** in a 24 day assay in plastic pots, for control (●), nitrogen CGC particles (●), 15 g L^{-1} FucoPol particles (●), 30 g L^{-1} FucoPol particles (●), and commercial fertilizer (●). **(c):** Pea growth in a 16 day assay in plastic pots for nitrogen CGC particles (●), 15 g L^{-1} FucoPol particles (●), and 30 g L^{-1} FucoPol particles (●).

In the CGC group only two seeds did germinate, and their growth was slower when compared with the control group. One of the plants started to die in the 22º day of plantation, and the other plant grow until the end (5.75 cm), but slower than in the other groups. In the plastic plot assay (Figure 3.13 b) both seeds germinated later when compared with the control group, in the seventh day and their growth was slower. One of the plants started to die in the 18º day, being similar to the tray assay, which seems that the quantity of CGC particles used was too much that the nitrogen amount could start to be inhibitory to the seed germination and growth, and in a later phase could even start to be toxic to the plant, and made it die. So another plastic pot assay was performed whit half the nitrogen CGC particles (Figure 3.13 c). Both seeds germinated later, in six days, and one of them died in the 13º day. The other growth was slow (4.5 cm).

In the FucoPol 15 g L^{-1} group all the seeds germinated and grew very fast until the end (11.4 cm) in the tray assay (Figure 3.13 a) and in the plastic plot grew 15 cm (Figure 3.13 b). In the FucoPol 30 g L^{-1} group only one of the seeds did not germinate, and the remaining grew very fast and they succeeded to grew higher (13.9 cm), in the plastic pot assay grew 14.5 cm. In the group of commercial fertilizer, only one seed did not germinate, and one started to die in the 20º day of plantation. The other two plants grew faster and higher (10.6 cm) and in the plastic pot grew 12.5 cm.

The nitrogen FucoPol particles had very similar growth ranges, and grew taller than the commercial fertilizer and the control plants. The growth profile between the nitrogen CGC and the FucoPol particles was very different in the tree assay, meaning that or nitrogen CGC particles was too much or too low to the initial plant growth.

The nitrogen FucoPol 15g L^{-1} and 30 g L^{-1} hydrogel particles (Figure 3.13 c) were used in the *in vivo* tests, where both seeds germinated in five days and grew fast and tall in 16 days (12.75 and 8.25 cm, respectively), being that the hydrogel form is also suitable to be used for plant growth.

It seems that the nitrogen FucoPol particles benefited plant growth, on the other hand for nitrogen CGC particles it was not right if it could be beneficial in a later growth plant stage, since they grow slower and one of the plants died in the tree assays. To be sure that nitrogen polymers particles influences on the plant nutrient necessities to grow, it would require an assay with many replicas.

The pea seeds had a fast germination and growth in general for all the groups. The plants early death (nitrogen CGC particles and commercial fertilizer) could had been due to the lake of space to growth and competition between the two plants for nutrients and maybe for lake of nutrients. Pictures where taken every day to the tray assay to follow the pea seeds germination and growth, and some are presented in Figure 3.14.

For all tests, the water released from the soil in the first day of plantation was collected and preserved for further analysis for ammonium and nitrate detection. The water collected from the CGC group) in the first day about 0.55 and 1.5 g L^{-1} of ammonium and nitrate, respectively

was release which is a higher amount than the estimated initial nitrogen content for 0.5 g of nitrogen CGC particles (0.07 g L^{-1}). Water was collected also in the 24º and 34º days, the ammonium concentration was lower in the day 24 and 34 (0.035 and 0.00 g L^{-1}, respectively) and nitrate was 0.4 g L^{-1}. At day 34 in was verified an increase in nitrate content (0.6 g L^{-1}), which may be due to the decomposition of the plant that died in the day 22. There was no trace of ammonium in the FucoPol samples, the nitrate present in the first day water samples was 0.008 g L^{-1} in the 15 g L^{-1} samples and 0.02 g L^{-1} for the 30gL^{-1} samples, which could be due to the higher nitrogen content in the 30 g L^{-1} particles. Control samples from the first day did not had traces of ammonium or nitrate, and the commercial fertilizer samples, only one of the replicas showed a low presence of nitrate (0.03 g L^{-1}).

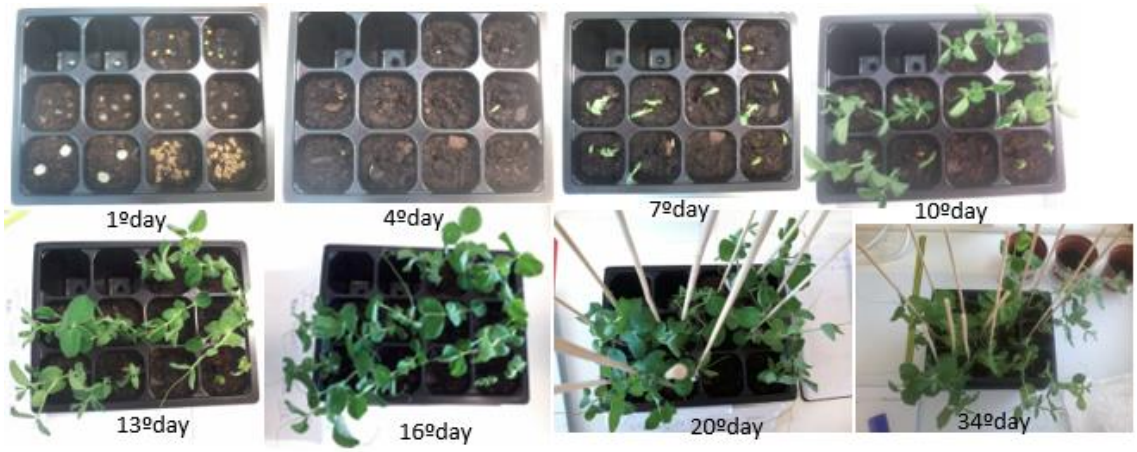

Figure 3.14. Pea germination and growth in a plant tray for 34 days, where is represented the first, fourth, seventh, thirteenth, sixteenth, twentieth and thirty-fourth days. In the first row from the left to the right control A, control B, CGC A, CGC B; in the middle row from the left to the right FucoPol 15 g L^{-1} A, FucoPol 15 g L^{-1} B, FucoPol 30 g L^{-1} A, FucoPol 30 g L^{-1} B; in the third row from the left to the right commercial fertilizer A and commercial fertilizer B.

At the end of the assay the plants were collected, cleaned and freeze-dried. After this the dried weight (%) was calculated. For the tray and plastic plot assays the control group had an average of 10 and 7 %, respectively, the commercial fertilizer of 11 and 8 %, which was relatively similar, may be due to the fact that the commercial fertilizer beads did not seem to degraded in soil, and so did not release nutrients to plant uptake (Table 3.7).

Table 3.7. Plant dried weight (%) for the different assays.

Assay	Sample	Plant Weight (g)	Hight (cm)	Freeze-dried Plant Weight (g)	Dried Weight (%)
Tray	Control	1.33 ± 0.27	12.3 ± 1.8	0.13 ± 0.05	10 ± 1
	CGC	1.02	11.5	0.13	13
	FucoPol 15 g L^{-1}	1.34 ± 0.53	11.4 ± 2.3	0.10 ± 0.04	7 ± 0
	FucoPol 30 g L^{-1}	1.60 ± 0.06	13.9 ± 2.0	0.19 ± 0.06	12 ± 4
	Commercial Fertilizer	2.18 ± 0.36	15.9 ± 0.5	0.26 ± 0.10	11 ± 3
Plastic Pots	Control	1.82	16.5	0.13	7
	CGC	1.25	9.3	0.19	16
	FucoPol 15 g L^{-1}	1.02 ± 0.46	15.0 ± 6.3	0.08 ± 0.04	8 ± 0
	FucoPol 30 g L^{-1}	1.42 ± 0.01	14.5 ± 2.0	0.12 ± 0.01	9 ± 1
	Commercial Fertilizer	0.97 ± 0.01	12.5 ± 1.7	0.08 ± 0.00	8 ± 0
	CGC A	0.98	9.0	0.12	12
	FucoPol A 15 g L^{-1}	1.37 ± 0.02	12.8 ± 0.3	0.13 ± 0.00	9 ± 0
	FucoPol A 30 g L^{-1}	1.03 ± 0.20	8.3 ± 2.3	0.10 ± 0.01	10 ± 1

The nitrogen CGC particles only survival plants for the tray and plastic pot assays was of 13 and 16 % and for the assay with half of the particles quantity was of 12 %, relatively higher than for the FucoPol 15 g L^{-1} (7 and 8 %) and 30 g L^{-1} (12 and 9 %) particles. The water content in these plants was high, and despite the fresh plant weight of the nitrogen CGC particles plant was relatively smaller (and did not growth taller as some) than the other group plants, its dried weight was higher, possibly had a superior protein, carbohydrates, among others, content (Table 3.7). Still the lack of CGC replicas in this assay does not allow to take these conclusions. It would be also interesting to see the nitrogen content of these plants, to see if there was nitrogen uptake differences for the particles, using for example the Kjeldahl method. It was tried to use the total nitrogen kit to perform this analysis, but it was not possible.

At the end of the assays, nitrogen CGC and FucoPol particles were not found which could mean that they degraded in the soil, so in a short period of time they may had released all the nitrogen nutrients. These particles for slow and controlled release formulations did not seem to be suitable, by the results obtained in the soil and water release tests. It could be due to the gradient differences between the particles concentrated with salt and the release medium, when in contact with the water or humid medium (soil) by diffusion the nutrients are released very rapidly for the lower salt medium, which explains the initial burst release. Also the soil microorganism's activity probably accelerated the polymers degradation. These polymers could be coated so that they would not be so susceptible to the diffusion and microorganism's activity. For example the hydrogel particles from FucoPol could be fortified with a more crosslinked matrix, by using a higher polymer concentration and/or using a higher concentration of crosslinker, as reported by Hussain *et al.* (2012). For the CGC particles, to optimize their controlled release properties, they could be coated with a polymer equally biodegradable but that could resist longer to the soil elements and avoid an excessive earlier nutrient release. Some of these formulations have been studied for example chitosan-starch beads (Perez and Francois, 2016), or chitosan and xanthan

with layered tablets formulation (Melaj and Daraio, 2013). Also the polymer chemical structure could be modified to improve its properties, for example, according to Kyzas and Bikiaris (2015) some of the modifications in chitosan chemical structure presented to be advantages for chitosan adsorption capacity, for example in grafting reactions are inserted additional functional groups, which leads to additional interaction sites, and so the adsorption capacity increases. On the other hand, the cross-linking reactions, where macromolecular chains are cross-linked, some functional groups (hydroxyl and amino groups) that are important for interaction with a dye or heavy metal are bonded to the cross-linker, decreasing the adsorption sites (Kyzas and Bikiaris, 2015).

4. Conclusions and Future Perspectives

In this thesis chitin-glucan complex (CGC) and FucoPol biopolymers were used to produce particles for controlled release of fertilizers formulations.

Nitrogen CGC particles were formed by saturated solutions of ammonium and nitrate salts and urea using 15, 20, 25 and 30 g L^{-1} of CGC, where the salts were uptaken by adsorption into the polymer particles for a 48 h period. The concentration of 15 g L^{-1} was the one that showed a more satisfactory salts uptake such as pH 5.0 in a range of pH 3.0, 5.0 and 8.0. Ammonium nitrate showed more satisfying results in terms of the polymer adsorption capacity at 15 g L^{-1} concentration. A 2 h contact between the CGC and the ammonium nitrate solution had a satisfactory nitrogen uptake of 22.55 % for a short period of time. These particles in water tests at 25 ºC had am initial burst release in just two days, and in soil it was also verified an initial burst release but relatively slower, in five days. The water medium is a more extreme medium than soil that could potentiate the nutrients burst release by the gradient differences between the polymer and water barriers.

Nitrogen FucoPol particles were formed by the production of hydrogel beads from a 10g L^{-1} solution of FucoPol with 15 and 30 g L^{-1} of ammonium nitrate using a 3.33 g L^{-1} iron (III) chloride hexahydrate solution as crosslinker. These particles had in fact nitrogen encapsulated, with a concentration of 0.343 g L^{-1} and 0.528 g L^{-1} for the 15 and 30 g L^{-1}, respectively. In water tests, the dried particles had a burst release in just one day, and in soil the burst release was verified in two days. The humid hydrogel particles in water had a burst release in two days, where the particles started to degrade.

These particles did not sim to fit in the slow and controlled release requirements when tested in water and in soil releasing, which seemed to have released the major part of ammonium and nitrate earlier. But the estimated initial content of the nitrogen CGC particles was low for the ammonium and nitrate content in the water and soil tests, which the real content must had been higher. For the nitrogen FucoPol particles, the initial estimated nitrogen content was higher than the released in the tests, so it wasn`t certain if the major nitrogen content had been released in the initial burst release or if some part had been still linked in the polymer matrix for both nitrogen FucoPol and CGC particles.

The tests with pea seeds in soil was satisfactory in general, the plants germination and growth was fast with the FucoPol particles for both ammonium nitrate concentrations (15 and 30 g L^{-1}) for the dried and humid hydrogel particles. With the nitrogen CGC particles a later germination and slower growth was verified, and some of the plants died earlier, even using half of the particles quantity. Still the quantity used could started to be toxic for the plant or it was a low quantity for the nutrients requirements for their initial growth. By the end of the assay there was no traces of the nitrogen FucoPol and CGC particles, they may had degraded.

As future perspectives, to optimize the controlled release properties of these particles, nitrogen should be coated with a polymer equally biodegradable but that could resist longer to the

soil elements and avoid an excessive earlier nutrient release. Some of these formulations have been studied for example chitosan-starch beads (Perez and Francois, 2016), or chitosan and xanthan with layered tablets formulation (Melaj and Daraio, 2013).

For future work, with the particles formulation optimized, and after the preliminary water and soil release tests, plant germination and grow should be performed with a greater number of samples per group for a longer period of time, so that the efficacy and efficiency of these particles in plant growth could be evaluated.

5. References

Akkaya G, Uzun İ, Güzel F. Kinetics of the adsorption of reactive dyes by chitin. Dyes Pigm 2007; 73: 168-177.

Alexandrova AN, Jorgensen WL. Why urea eliminates ammonium rather than hydrolyzes in aqueous solution. J Phys Chem B 2007; 111: 720-730.

Alves VD, Freitas F, Torres CAV, Cruz M, Marques R, Grandfils C, Gonçalves MP, Oliveira R, Reis MAM. Rheological and morphological characterization of the culture broth during exopolysccharide production by *Enterobacter sp.* Carbohydr Polym 2010; 81: 758-764.

Campos EVR, Oliveira JL, Fraceto LF, Singh B. Polysaccharides as safer release systems for agrochemicals. Agron Sustain Dev 2015; 35: 47-66.

Chagas B, Farinha I, Galinha CF, Freitas F, Reis MAM. Chitin-glucan complex production by *Komagataella* (*Pichia*) *pastoris*: impact of cultivation pH and temperature on polymer content and composition. N Biotechnol 2014; 31: 468-474.

Davidson D, Gu FX. Materials for sustained and controlled release of nutrients and molecules to support plant growth. J Agric Food Chem 2012; 60:870-876.

Du WL, Xu ZR, Han XY, Xu YL, Miao ZG. Preparation, characterization and adsorption properties of chitosan nanoparticles for eosin Y as a model anionic dye. J Hazard Mater 2008; 153: 152-156.

Farinha I, Duarte P, Pimentel A, Plotnikova E, Chagas B, Mafra L, Grandfils C, Freitas F, Fortunato E, Reis MAM. Chitin-glucan complex production by *Komagataella pastoris*: downstream optimization and product characterization. Carbohydr Polym 2015; 130: 455-464.

Farinha I, Freitas F, Reis MAM. Implementation of a repeated fed-batch process for the production of chitin-glucan complex by *Komagataella pastoris*. N Biotechnol 2016; 37: 123-128.

Fertilizers Europe. “Quality first”: position paper of Fertilizers Europe on EU Commission proposal for new regulation on availability of CE marked fertilizing products [COM(2016)157] of 17 March 2016. Brussels. 2016; 1-11.

Fialho L. Preparation of biopolymer structures based on FucoPol. Master thesis, FCT-UNL 2017.

Food and Agriculture Organization of the United Nations (FAO). World fertilizer trends and outlook to 2018. Rome. 2015; 1-53.

Freitas F, Alves VD, Gouveia AR, Pinheiro C, Torres CAV, Grandfils C, Reis MAM. Controlled production of exopolysaccharides from *Enterobacter* A47 as a function of carbon source with demonstrations of their film and emulsifying abilities. Appl Biochem Biotechnol 2014; 172: 641-657.

Freitas F, Alves VD, Torres CAV, Cruz M, Sousa I, Melo MJ, Ramos AM, Reis MAM. Fucose-containing exopolysaccharide produced by the newly isolated *Enterobacter* strain A47 DSM 23139. Carbohydr Polym 2011; 83: 159-165.

Guilherme MR, Aouada FA, Fajardo AR, Martins AF, Paulino AT, Davi MFT, Rubira AF, Muniz EC. Superabsorbent hydrogels based on polysaccharide for application in agriculture as soil conditioner and nutrient carrier: a review. Eur Polym J 2015; 72: 365-385.

Heffer P, Prud`homme M. Fertilizer Outlook 2015-2019. Istanbul. International Fertilizer Industry Association 2015; 1-6. Arab J Chem 2011; 4: 389-395.

Hussain MR, Devi RR, Maji TK. Controlled release of urea from chitosan microspheres prepared by emulsification and cross-linking method. Iran Polym J 2012; 21: 473-479.

Iqbal J, Wattoo FH, Wattoo MHS, Malik R, Tirmizi SA, Imran M, Ghangro AB. Adsorption of acid yellow dye on flakes of chitosan prepared from fishery wastes. Arab J Chem 2011; 4: 389-395.

Jamnongkan T, Kaewpirom S. Potassium release kinetics and water retention of controlled-release fertilizers based on chitosan hydrogels. J Polym Environ 2010; 18: 413-421.

Kashyap PL, Xiang X, Heiden P. Chitosan nanoparticle based delivery systems for sustainable agriculture. Int J Biol Macromolec 2015; 77: 36-51.

Kyzas GZ, Bikiaris DN. Recent modifications of chitosan for adsorption applications: a critical and systematic review. Mar Drugs 2015; 13: 312-337.

Li X, Li Q, Xu X, Su Y, Yue Q, Gao B. Characterization, swelling and slow-release properties of a new controlled release fertilizer based on wheat straw cellulose hydrogel. J Taiwan Inst Chem Eng 2016; 60: 564-572.

Lin CC, Metters AT. Hydrogels in controlled release formulation: network design and mathematical modeling. Adv Drug Deliv Rev 2006; 58: 1379-1408.

Liu Z, Jiao Y, Wang Y, Zhou C, Zhang Z. Polysaccharides-based nanoparticles as drug delivery systems. Adv Drug Deliv Rev 2008; 60: 1650-1662.

Longhinotti E, Pozza F, Furlan L, Sanchez MNM, Klug M, Laranjeira MCM, Fávere VT. Adsorption of anionic dyes on the biopolymer chitin. J Braz Chem Soc 1998; 9: 435-440.

Mayer H. Nutrient release patterns of controlled release fertilizers used in the ornamental horticulture industry of South Florida. Master Thesis, University of Florida 2010; 1-79.

Melaj MA, Daraio ME. Preparation and characterization of potassium nitrate controlled-release fertilizers based on chitosan and xanthan layered tablets. J Appl Polym Sci 2013; 130: 2422-2428.

Minet EP, O`Carroll C, Rooney D, Breslin C, McCarthy CP, Gallagher L, Richards KG. Slow delivery of a nitrification inhibitor (dicyandiamide) to soil using a biodegradable hydrogel of chitosan. Chemosphere 2013; 93: 2854-2858.

Montesano FF, Parente A, Santamaria P, Sannino A, Serio F. Biodegradable superabsorbent hydrogel increases water retention properties of growing media and plant growth. Agric Agric Sci Procedia 2015; 4: 451-458.

Ngah WSW, Kamari A, Fatinathan S, Ng PW. Adsorption of chromium from aqueous solution using chitosan beads. Adsorption 2006; 12: 249-257.

Perez JJ, Francois NJ. Chitosan-starch beads prepared by ionotropic gelation as potential matrices for controlled release of fertilizers. Carbohydr Polym 2016; 148: 134-142.

Raemdonck K, Martens TF, Braeckmans K, Demeester J, De Smedt SC. Polysaccharide-based nucleic acid nanoformulations. Adv Drug Deliv Rev 2013; 65: 1123-1147.

Roca C, Chagas B, Farinha I, Freitas F, Mafra L, Aguiar F, Oliveira R, Reis MAM. Production of yeast chitin-glucan complex from biodiesel industry byproduct. Process Biochem 2012; 47:1670-1675.

Roshanravan B, Soltani SM, Rashid SA, Mahdavi F, Yusop MK. Enhancement of nitrogen release properties of urea-kaolinite fertilizer with chitosan binder. Chem Spec Bioavailab 2015; 27: 44-51.

Saha TK, Bhoumik NC, Karmaker S, Ahmed MG, Ichikawa H, Fukumori Y. Adsorption of methyl orange onto chitosan from aqueous solution. J Water Resource Prot 2010; 2: 898-906.

Sempeho SI, Kim HT, Mubofu E, Pogrebnoi A, Shao G, Hilonga A. Encapsulated urea-kaolinite nanocomposite for controlled-release fertilizer formulations. J Chem 2015; 2015: 1-17.

Shaviv A. Advances in controlled release of fertilizers. Adv Agron 2000; 71: 1-49.

Timilsena YP, Adhikari R, Casey P, Muster T, Gill H, Adhikari B. Enhanced efficiency fertilisers: a review of formulation and nutrient release patterns. J Sci Food Agric 2014; 95:1131-1142.

Torres CAV, Ferreira ARV, Freitas F, Reis MAM, Coelhoso I, Sousa I, Alves VD. Rheological studies of the fucose-rich exopolysaccharide FucoPol. Int J Biol Macromol 2015; 79: 611-617.

Torres CAV, Marques R, Antunes S, Alves VD, Sousa I, Ramos AM, Oliveira R, Freitas F, Reis MAM. Kinetics of production and characterization of the fucose-containing exopolysaccharide from *Enterobacter* A47. J Biotechnol 2011; 156: 261-267.

Trenkel ME. Slow- and controlled-release and stabilized fertilizers: an option for enhancing nutrient use efficiency in agriculture. Paris. International Fertilizer Industry Association 2010; 8-133.

Trinh TH, Dao QN, KuShaari K, Ismail L, Shuib A. Study the release of urea Agrium® coated urea using UV-Vis spectrometer. Appl Mech Mater 2014: 625: 745-748.

Trinh TH, KuShaari K, Shuib A, Ismail L, Azeem B. Modelling the release of nitrogen from controlled release fertiliser: constant and decay release. Biosyst Eng 2015; 130: 34-42.

Vundavalli R, Vundavalli S, Nakka M, Rao DS. Biodegradable nano-hydrogels in agricultural framing - alternative source for water resources. Procedia Mater Sci 2015; 10: 548-554.

Wong YC, Szeto YS, Cheung WH, McKay G. Adsorption of acid dyes on chitosan – equilibrium isotherm analyses. Process Biochem 2004; 39: 693-702.

Yamamoto CF, Pereira EI, Mattoso LHC, Matsunaka T, Ribeiro C. Slow release fertilizers based on urea/urea-formaldehyde polymer nanocomposites. Chem Eng J 2016; 287: 390-397.

Zhou D, Zhang L, Zhou J, Guo S. Cellulose/chitin beads for adsorption of heavy metals in aqueous solutions. Water Res 2004; 38:2643-2650.

Appendix

A.1. Commercial Fertilizer Composition

The commercial fertilizer was from Siro, and was a controlled-release fertilizer that provides nutrients during six months, especially for garden and horticultural plants. It was presented in the form of rigid beads. The composition is described in Table A.1.

Table A.1. SIRO commercial fertilizer chemical composition.

Composition	**%**
Total Nitrogen (N)	18.0
Ammoniacal	1.3
Ureic	16.8
Phosphorus pentoxide (P_2O_5)	6.0
Potassium oxide (K_2O)	8.0
Sulfuric oxide (SO_3)	8.5
Free Sulphur	3.4
Magnesium oxide (MgO)	4.2
Free Magnesium	2.5